

## P-GLYCOPROTEINS AND USES THEREOF

### Related Applications

This application claims priority under 35 U.S.C. §119 from U.S. provisional patent  
5 application 60/156,510, filed September 28, 1999.

### Field of the Invention

The invention pertains to P-glycoproteins of dog (*Canis familiaris*).

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### Background of the Invention

P-glycoprotein (PGP; also known as multidrug transporter, MDR1) is a member of the ABC transporter superfamily and is expressed in the human intestine, liver and other tissues. This enzyme serves as an efflux pump exporting small molecules across the cell membrane. It has been known for several years that high level expression of PGP is a mechanism for tumor resistance to cancer chemotherapy. Intestinal expression of PGP may affect the oral bioavailability of drug molecules that are substrates for this transporter. PGP can efficiently efflux drugs back into the intestinal lumen and thus reduce the amount of drug that enters into circulation.

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The measurement of interaction with PGP can provide a better understanding of the reasons why particular drugs demonstrate low or high bioavailability. Interaction with PGP can be studied using either direct assays of drug transport in polarized cell systems or with indirect assays such as drug-stimulated ATPase activity and inhibition of the transport of fluorescent substrates.

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Therefore there is a need for additional PGP polypeptides, preferably which are closely related to the human PGP, for use in the foregoing drug assays.

### Summary of the Invention

Nucleic acids encoding P-glycoprotein of dog (*Canis familiaris*) have now been identified, isolated, cloned and sequenced. This PGP is closely related (has a high degree of identity) to the human PGP. The invention provides isolated nucleic acid molecules, unique fragments of those molecules, expression vectors containing the foregoing, and host cells transfected with those molecules. The invention also provides isolated polypeptides and

inhibitors of the foregoing nucleic acids and polypeptides which reduce drug transport. The PGP nucleic acids and polypeptides are useful in assays for evaluating bioavailability of drugs, as well as for the optimization or discovery of drugs. In addition, the foregoing can be used in the diagnosis or treatment of conditions characterized by PGP activity and can be used 5 in methods in which it is therapeutically useful to increase or decrease PGP activity.

According to one aspect of the invention, an isolated nucleic acid molecule is provided which is selected from the group consisting of (a) nucleic acid molecules that code for the amino acid sequence of SEQ ID NO:2, (b) allelic variants of (a), wherein the allelic variants exclude SEQ ID NO:3 and SEQ ID NO:5, and (c) complements of (a) or (b).

10 Preferred allelic variants include nucleic acid molecules that encode an amino acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:25 and SEQ ID NO:27, particularly nucleotide sequences selected from the group consisting of SEQ ID NO:22, SEQ ID NO:24 and SEQ ID NO:26. In preferred embodiments the isolated nucleic acid molecule codes for SEQ ID NO:2, or comprises the nucleotide sequence of SEQ ID NO:1. In 15 particularly preferred embodiments, the nucleic acid molecules comprise or consist of the coding region of the aforementioned nucleotide sequences.

According to other aspects of the invention, isolated P-glycoprotein polypeptides or fragments thereof are provided. The polypeptides include at least one amino acid of a dog P-glycoprotein selected from the group consisting of amino acids 25, 192, 197, 212, 288, 329, 20 532, 696, 1273 and 1355 of SEQ ID NO:2; amino acid 25 of SEQ ID NO:23; and amino acids 25 and 1148 of SEQ ID NO:25. Other isolated P-glycoprotein polypeptides or fragments thereof include at least one amino acid of a dog P-glycoprotein selected from the group consisting of amino acids 3, 6, 8, 10, 12, 14-26, 36, 38, 48, 52, 56, 64, 74, 78, 84-92, 94, 96, 98, 99, 101, 103, 104, 106, 108, 112, 115, 147, 187, 197, 199, 233, 288, 321, 326, 347, 397, 25 450, 454, 455, 467, 472, 520, 633, 637, 643, 644, 650, 657, 658, 661, 666, 667, 674-677, 679, 685, 689, 691, 693, 694, 703, 707, 717, 731, 736, 740, 744, 745, 756, 759, 763, 853, 914, 920, 942, 943, 946, 968-970, 972, 974, 983, 1005, 1010, 1017, 1025, 1026, 1029, 1040, 1095, 1098, 1105, 1144, 1148, 1149, 1158, 1162, 1165, 1168, 1170, 1252 and 1279 of SEQ ID NO:2; and amino acid 329 of SEQ ID NO:27, wherein the P-glycoprotein is identical to a 30 human P-glycoprotein except for the at least one amino acid of a dog P-glycoprotein. In certain embodiments, the human P-glycoprotein is selected from the group of SEQ ID NO:7 and SEQ ID NO:8. Still other isolated P-glycoprotein polypeptides or fragments thereof

include at least one amino acid of a dog P-glycoprotein that is different from a cynomolgous P-glycoprotein, wherein the P-glycoprotein is identical to a cynomolgous monkey P-glycoprotein except for the at least one amino acid of a dog P-glycoprotein. Yet other polypeptides include combinations of the foregoing dog, human and cynomolgous PGP polypeptides. In preferred embodiments, the isolated P-glycoprotein polypeptides or fragments thereof include an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, fragments of SEQ ID NO:2, fragments of SEQ ID NO:23, fragments of SEQ ID NO:25 and fragments of SEQ ID NO:27.

According to still other embodiments of the invention, isolated nucleic acid molecules are provided which encode the foregoing isolated P-glycoprotein polypeptides or fragments thereof. Also included expression vectors comprising the foregoing isolated nucleic acid molecules operably linked to a promoter, as well as host cells transformed or transfected with the expression vectors.

In another aspect of the invention, agents which selectively binds the isolated PGP polypeptides are provided. Preferably the agent does not bind a human or dog P-glycoprotein, except those provided herein. In certain embodiments, the agent is a polypeptide preferably one selected from the group consisting of monoclonal antibodies, polyclonal antibodies, Fab antibody fragments, F(ab)<sub>2</sub> antibody fragments and antibody fragments including a CDR3 region. Also provided are agents which selectively binds the foregoing isolated nucleic acid molecules, preferably antisense nucleic acid molecules which selectively binds to the isolated nucleic acid molecule.

According to another aspect of the invention, methods for predicting the bioavailability of a compound are provided. The methods include measuring the transmembrane transport of a test compound by a first P-glycoprotein, comparing the transmembrane transport of the test compound by the first P-glycoprotein and a second P-glycoprotein to predict the bioavailability of the test compound, wherein the relative amount or rate of transport by the first P-glycoprotein and the second P-glycoprotein is predictive of bioavailability of the test compound. In certain embodiments the first P-glycoprotein is selected from the group consisting of dog P-glycoproteins and primate P-glycoproteins, preferably one of the foregoing polypeptides. In other embodiments the second P-glycoprotein is a human P-glycoprotein.

In still other aspects of the invention, methods for inhibiting P-glycoprotein

transporter activity in a mammalian cell are provided. The methods include contacting the mammalian cell with an amount of one of the foregoing agents effective to inhibit P-glycoprotein transporter activity in the mammalian cell.

Also included in the invention are methods for increasing bioavailability of a drug in a subject. The methods include administering to a subject in need of such treatment one of the foregoing agents in an amount effective to increasing bioavailability of a drug. The inhibitor can be administered prior to administering the drug, or concurrently with the drug.

Also provided are methods for increasing P-glycoprotein transporter activity in a cell. These methods include contacting the cell with a molecule selected from the group consisting of the foregoing nucleic acid molecules, in an amount effective to increase P-glycoprotein transporter activity in the cell. The cell can be contacted under conditions whereby the P-glycoprotein is expressed.

According to yet another aspect of the invention, methods for identifying lead compounds for a pharmacological agent useful in the treatment of disease associated with P-glycoprotein transporter activity are provided. The methods include providing a cell or other membrane-encapsulated space comprising a P-glycoprotein as provided herein; contacting the cell or other membrane-encapsulated space with a candidate pharmacological agent under conditions which, in the absence of the candidate pharmacological agent, cause a first amount of P-glycoprotein transporter activity; and determining a second amount of P-glycoprotein transporter activity as a measure of the effect of the pharmacological agent on the P-glycoprotein transporter activity, wherein a second amount of P-glycoprotein transporter activity which is less than the first amount indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which reduces P-glycoprotein transporter activity and wherein a second amount of P-glycoprotein transporter activity which is greater than the first amount indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which increases P-glycoprotein transporter activity. The methods can further include a step of loading the cell or other membrane-encapsulated space with a detectable compound, wherein the compound is detected as a measure of the P-glycoprotein transporter activity.

Also included are methods for identifying compounds which selectively bind a P-glycoprotein. The methods include contacting a P-glycoprotein provided herein with a compound, and determining the binding of the compound to the P-glycoprotein. The methods

can further include determining the effect of the compound on the P-glycoprotein transporter activity of the P-glycoprotein or determining the effect of the compound on the ATPase activity of the P-glycoprotein.

Additional methods provided according to the invention include methods for

5 determining ATPase activity of a P-glycoprotein. The methods include contacting a host cell as provided above, or a membrane fraction thereof, with a test drug, and measuring ATPase activity of the P-glycoprotein. In certain embodiments, the step of measuring ATPase activity is performed at least twice at different times. Also provided methods for determining transmembrane transport of a compound by a P-glycoprotein. The methods include

10 contacting a host cell provided above, or a membrane fraction thereof, with a test drug, and measuring transport of the test drug under sink conditions in at least one direction of transport selected from the group consisting of the apical to basolateral direction and the basolateral to apical direction. In certain embodiments the step of measuring transport of the test drug is performed at least twice at different times.

15 These and other aspects of the invention are described in greater detail below.

#### Brief Description of the Sequences

SEQ ID NO:1 is the nucleotide sequence encoding dog P-glycoprotein (Genotype C).

SEQ ID NO:2 is the amino acid sequence of a dog P-glycoprotein encoded by SEQ ID NO:1.

SEQ ID NO:3 is the nucleotide sequence encoding a prior art dog P-glycoprotein (GenBank accession number AF045016).

SEQ ID NO:4 is the amino acid sequence of a dog P-glycoprotein encoded by SEQ ID NO:3.

25 SEQ ID NO:5 is the nucleotide sequence encoding another prior art dog P-glycoprotein (GenBank accession number AF092810).

SEQ ID NO:6 is the amino acid sequence of a dog P-glycoprotein encoded by SEQ ID NO:5.

30 SEQ ID NO:7 is the amino acid sequence of a prior art human P-glycoprotein, having Genbank accession number M14758.

SEQ ID NO:8 is the amino acid sequence of a second prior art human P-glycoprotein, having Genbank accession numbers AF016535 or NM\_000927.

SEQ ID NO:9 is the nucleotide sequence of a PCR primer homologous to human PGP1.

SEQ ID NO:10 is the nucleotide sequence of a PCR primer homologous to dog PGP2.

SEQ ID NO:11 is the nucleotide sequence of a PCR primer homologous to dog PGP1.

5 SEQ ID NO:12 is the nucleotide sequence of a PCR primer homologous to human PGP1.

SEQ ID NO:13 is the nucleotide sequence of a PCR primer homologous to dog PGP1.

SEQ ID NO:14 is the nucleotide sequence of a PCR primer homologous to human PGP1.

10 SEQ ID NO:15 is the nucleotide sequence of a PCR primer homologous to dog PGP1.

SEQ ID NO:16 is the nucleotide sequence of a PCR primer homologous to human PGP1.

SEQ ID NO:17 is the nucleotide sequence of a PCR primer homologous to dog PGP1.

SEQ ID NO:18 is the nucleotide sequence of a PCR primer homologous to vector.

15 SEQ ID NO:19 is the nucleotide sequence of a PCR primer homologous to dog PGP1.

SEQ ID NO:20 is the nucleotide sequence of a PCR primer homologous to human PGP1.

SEQ ID NO:21 is the nucleotide sequence of a PCR primer homologous to dog PGP1.

SEQ ID NO:22 is the nucleotide sequence encoding dog P-glycoprotein (Genotype A).

20 SEQ ID NO:23 is the amino acid sequence of a dog P-glycoprotein encoded by SEQ ID NO:22.

SEQ ID NO:24 is the nucleotide sequence encoding dog P-glycoprotein (Genotype B).

SEQ ID NO:25 is the amino acid sequence of a dog P-glycoprotein encoded by SEQ ID NO:24.

25 SEQ ID NO:26 is the nucleotide sequence encoding dog P-glycoprotein (Genotype D).

SEQ ID NO:27 is the amino acid sequence of a dog P-glycoprotein encoded by SEQ ID NO:25.

SEQ ID NO:28 is the nucleotide sequence of the cDNA synthesis primer from the Marathon cDNA Amplification Kit.

30 SEQ ID NO:29 is the nucleotide sequence of the cDNA Adapter Primer 1 from the Marathon cDNA Amplification Kit.

SEQ ID NO:30 is the nucleotide sequence of a PCR primer homologous to dog PGP1.

SEQ ID NO:31 is the nucleotide sequence of a PCR primer homologous to dog PGP1.

SEQ ID NO:32 is the nucleotide sequence of a PCR primer homologous to dog PGP1.

**Detailed Description of the Invention**

5 The present invention in one aspect involves the identification of novel cDNAs encoding dog P-glycoproteins, referred to herein as dog PGP. The nucleotide sequence of a dog PGP (termed “Genotype C”) is presented as SEQ ID NO:1, and the amino acid sequence of this dog PGP is presented as SEQ ID NO:2.

Three allelic variants of Genotype C dog PGP were isolated (“Genotype A”,  
10 “Genotype B” and “Genotype D”). The nucleotide and amino acid sequences of Genotype A are presented as SEQ ID NOs:20 and 21, respectively. The amino acid sequence of Genotype A differs from the amino acid sequence of Genotype C at amino acid 197 (histidine → glutamine) based on a C → A change at nucleotide 607 of SEQ ID NO:2. The nucleotide and amino acid sequences of Genotype B are presented as SEQ ID NOs:22 and 23, respectively.  
15 The amino acid sequence of Genotype B differs from the amino acid sequence of Genotype C at amino acid 25 (asparagine → lysine) based on a T → A change at nucleotide 91 of SEQ ID NO:2, and at amino acid 197 (histidine → glutamine) based on a C → A change at nucleotide 607 of SEQ ID NO:2. The nucleotide and amino acid sequences of Genotype D are presented as SEQ ID NOs:24 and 25, respectively. The amino acid sequence of Genotype D differs  
20 from the amino acid sequence of Genotype C at amino acid 25 (asparagine → lysine) based on a T → A change at nucleotide 91 of SEQ ID NO:2, at amino acid 197 (histidine → glutamine) based on a C → A change at nucleotide 607 of SEQ ID NO:2, at amino acid 329 (serine → threonine) based on a T → A change at nucleotide 1001 of SEQ ID NO:2, and at amino acid 1148 (methionine → valine) based on a A → G change at nucleotide 3458 of SEQ  
25 ID NO:2.

Two closely related dog PGP sequence were deposited in GenBank under accession numbers AF045016 (complete cDNA) and AF092810 (partial cDNA). Whereas much of the polypeptides presented herein is identical to the known dog PGPs, the dog PGPs of the invention (SEQ ID NOs:2, 21, 23 and 25) have several single amino acid differences from the prior art sequences, including at least one deletion. These allelic differences in the very highly conserved protein domains of the P-glycoprotein are entirely unexpected.

The invention involves in one aspect dog PGP nucleic acids and polypeptides, as well

as therapeutics relating thereto. The invention also embraces isolated functionally equivalent variants, useful analogs and fragments of the foregoing nucleic acids and polypeptides; complements of the foregoing nucleic acids; and molecules which selectively bind the foregoing nucleic acids and polypeptides.

5        The dog PGP nucleic acids and polypeptides of the invention are isolated. As used herein with respect to nucleic acids, the term "isolated" means: (i) amplified *in vitro* by, for example, polymerase chain reaction (PCR); (ii) recombinantly produced by cloning; (iii) purified, as by cleavage and gel separation; or (iv) synthesized by, for example, chemical synthesis. An isolated nucleic acid is one which is readily manipulable by recombinant DNA  
10      techniques well known in the art. Thus, a nucleotide sequence contained in a vector in which 5' and 3' restriction sites are known or for which polymerase chain reaction (PCR) primer sequences have been disclosed is considered isolated but a nucleic acid sequence existing in its native state in its natural host is not. An isolated nucleic acid may be substantially purified, but need not be. For example, a nucleic acid that is isolated within a cloning or  
15      expression vector is not pure in that it may comprise only a tiny percentage of the material in the cell in which it resides. Such a nucleic acid is isolated, however, as the term is used herein because it is readily manipulable by standard techniques known to those of ordinary skill in the art. An isolated nucleic acid as used herein is not a naturally occurring chromosome.

20        As used herein with respect to polypeptides, "isolated" means separated from its native environment and present in sufficient quantity to permit its identification or use. Isolated, when referring to a protein or polypeptide, means, for example: (i) selectively produced by expression cloning or (ii) purified as by chromatography or electrophoresis. Isolated proteins or polypeptides may be, but need not be, substantially pure. The term "substantially pure" means that the proteins or polypeptides are essentially free of other  
25      substances with which they may be found in nature or *in vivo* systems to an extent practical and appropriate for their intended use. Substantially pure polypeptides may be produced by techniques well known in the art. Because an isolated protein may be admixed with a pharmaceutically acceptable carrier in a pharmaceutical preparation, the protein may comprise only a small percentage by weight of the preparation. The protein is nonetheless isolated in  
30      that it has been separated from the substances with which it may be associated in living systems, i.e. isolated from other proteins.

As used herein a dog PGP nucleic acid refers to an isolated nucleic acid molecule

which codes for a dog PGP polypeptide. Such nucleic acid molecules code for dog PGP polypeptides which include the amino acid sequences of SEQ ID NO:2, 23, 25, 27 and fragments thereof. The nucleic acid molecules include the nucleotide sequences of SEQ ID NO:1, and nucleotide sequences which differ from the sequences of SEQ ID NO:1, in codon sequence due to the degeneracy of the genetic code. The dog PGP nucleic acids of the invention also include alleles (e.g., SEQ ID Nos: 22, 24, 26) of the foregoing nucleic acids (except those of previously known dog PGP alleles, e.g., SEQ ID NOS:3 and 5), and encoded polypeptides, as well as fragments of the foregoing nucleic acids and polypeptides. Such fragments can be used, for example, as probes in hybridization assays and as primers in a polymerase chain reaction (PCR). Preferred dog PGP nucleic acids include the nucleic acid sequences of SEQ ID NOS:1, 22, 24, 26 and fragments thereof. Complements of the foregoing nucleic acids also are embraced by the invention.

As used herein "dog PGP activity" refers to an ability of a PGP polypeptide to export small molecules across the cell membrane. A molecule which inhibits dog PGP activity (an antagonist) is one which inhibits export of small molecules via PGP and a molecule which increases dog PGP activity (an agonist) is one which increases export of small molecules via PGP. Changes in dog PGP activity can be measured by assays such as those disclosed herein, including efflux of fluorescent compounds from cells.

Alleles of the dog PGP nucleic acids of the invention can be identified by conventional techniques. For example, alleles of dog PGP can be isolated by hybridizing a probe which includes at least a fragment of SEQ ID NO:1 under stringent conditions with a cDNA library and selecting positive clones. Thus, an aspect of the invention is those nucleic acid sequences which code for dog PGP polypeptides and which hybridize to a nucleic acid molecule consisting of SEQ ID NO:1 under stringent conditions (except those of previously known dog PGP alleles, e.g., SEQ ID NOS:3 and 5). The term "stringent conditions" as used herein refers to parameters with which the art is familiar. Nucleic acid hybridization parameters may be found in references which compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. More specifically, stringent conditions, as used herein, refers, for example, to hybridization at 65°C in hybridization buffer (3.5 x SSC, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% Bovine Serum Albumin, 2.5mM

NaH<sub>2</sub>PO<sub>4</sub>(pH7), 0.5% SDS, 2mM EDTA). SSC is 0.15M sodium chloride/0.15M sodium citrate, pH7; SDS is sodium dodecyl sulphate; and EDTA is ethylenediaminetetraacetic acid. After hybridization, the membrane upon which the DNA is transferred is washed at 2 x SSC at room temperature and then at 0.1 - 0.5 x SSC/0.1 x SDS at temperatures up to 68°C.

5 There are other conditions, reagents, and so forth which can be used, which result in a similar degree of stringency. The skilled artisan will be familiar with such conditions, and thus they are not given here. It will be understood, however, that the skilled artisan will be able to manipulate the conditions in a manner to permit the clear identification of alleles of dog PGP nucleic acids of the invention. The skilled artisan also is familiar with the  
10 methodology for screening cells and libraries for expression of such molecules which then are routinely isolated, followed by isolation of the pertinent nucleic acid molecule and sequencing.

15 In screening for dog PGP nucleic acids, a Southern blot may be performed using the foregoing stringent conditions, together with a radioactive probe. After washing the membrane to which the DNA is finally transferred, the membrane can be placed against X-ray film to detect the radioactive signal.

20 The dog PGP nucleic acids of the invention also include degenerate nucleic acids which include alternative codons to those present in the native materials. For example, serine residues are encoded by the codons TCA, AGT, TCC, TCG, TCT and AGC. Each of the six codons is equivalent for the purposes of encoding a serine residue. Thus, it will be apparent to one of ordinary skill in the art that any of the serine-encoding nucleotide triplets may be employed to direct the protein synthesis apparatus, *in vitro* or *in vivo*, to incorporate a serine residue into an elongating dog PGP polypeptide. Similarly, nucleotide sequence triplets which encode other amino acid residues include, but are not limited to: CCA, CCC, CCG and  
25 CCT (proline codons); CGA, CGC, CGG, CGT, AGA and AGG (arginine codons); ACA, ACC, ACG and ACT (threonine codons); AAC and AAT (asparagine codons); and ATA, ATC and ATT (isoleucine codons). Other amino acid residues may be encoded similarly by multiple nucleotide sequences. Thus, the invention embraces degenerate nucleic acids that differ from the biologically isolated nucleic acids in codon sequence due to the degeneracy of  
30 the genetic code.

The invention also provides modified nucleic acid molecules which include additions, substitutions and deletions of one or more nucleotides. In preferred embodiments, these

modified nucleic acid molecules and/or the polypeptides they encode retain at least one activity or function of the unmodified nucleic acid molecule and/or the polypeptides, such as transporter activity, etc. In certain embodiments, the modified nucleic acid molecules encode modified polypeptides, preferably polypeptides having conservative amino acid substitutions

5 as are described elsewhere herein. The modified nucleic acid molecules are structurally related to the unmodified nucleic acid molecules and in preferred embodiments are sufficiently structurally related to the unmodified nucleic acid molecules so that the modified and unmodified nucleic acid molecules hybridize under stringent conditions known to one of skill in the art.

10 For example, modified nucleic acid molecules which encode polypeptides having single amino acid changes can be prepared. Each of these nucleic acid molecules can have one, two or three nucleotide substitutions exclusive of nucleotide changes corresponding to the degeneracy of the genetic code as described herein. Likewise, modified nucleic acid molecules which encode polypeptides having two amino acid changes can be prepared which have, e.g., 2-6 nucleotide changes. Numerous modified nucleic acid molecules like these will be readily envisioned by one of skill in the art, including for example, substitutions of nucleotides in codons encoding amino acids 2 and 3, 2 and 4, 2 and 5, 2 and 6, and so on. In the foregoing example, each combination of two amino acids is included in the set of modified nucleic acid molecules, as well as all nucleotide substitutions which code for the

15 amino acid substitutions. Additional nucleic acid molecules that encode polypeptides having additional substitutions (i.e., 3 or more), additions or deletions (e.g., by introduction of a stop codon or a splice site(s)) also can be prepared and are embraced by the invention as readily envisioned by one of ordinary skill in the art. Any of the foregoing nucleic acids or

20 polypeptides can be tested by routine experimentation for retention of structural relation or activity to the nucleic acids and/or polypeptides disclosed herein.

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The invention also provides isolated fragments of SEQ ID NO:1. The fragments can be used as probes in Southern blot assays to identify such nucleic acids, or can be used in amplification assays such as those employing PCR. Smaller fragments are those comprising 12, 13, 14, 15, 16, 17, 18, 20, 22, 25, 30, 40, 50, or 75 nucleotides, and every integer therebetween, and are useful e.g. as primers for nucleic acid amplification procedures. As known to those skilled in the art, larger probes such as 200, 250, 300, 400 or more nucleotides are preferred for certain uses such as Southern blots, while smaller fragments will be preferred

for uses such as PCR. Fragments also can be used to produce fusion proteins for generating antibodies or determining binding of the polypeptide fragments. Likewise, fragments can be employed to produce non-fused fragments of the dog PGP polypeptides, useful, for example, in the preparation of antibodies, in immunoassays, and the like. The foregoing nucleic acid  
5 fragments further can be used as antisense molecules to inhibit the expression of dog PGP nucleic acids and polypeptides, particularly for therapeutic purposes as described in greater detail below.

The invention also includes functionally equivalent variants of the dog PGP, which include variant nucleic acids and polypeptides which retain one or more of the functional  
10 properties of the dog PGP. For example, variants include a fusion protein which includes the extracellular and transmembrane domains of the dog PGP which retains the ability to transport molecules. Still other functionally equivalent variants include truncations, deletions, point mutations, or additions of amino acids to the sequence of SEQ ID NO:1 which retains functions of SEQ ID NOs:2, e.g., the alleles presented herein. Functionally equivalent  
15 variants also include a dog PGP which has had a portion of the N-terminus removed or replaced by a similar domain from another P-glycoprotein (e.g. a “domain-swapping” variant). Other functionally equivalent variants will be known to one of ordinary skill in the art, as will methods for preparing such variants. The activity of a functionally equivalent variant can be determined using the methods provided herein, and in references that have  
20 described assays using P-glycoproteins of other species. Such variants are useful, *inter alia*, for evaluating bioavailability of drugs, in assays for identification of compounds which bind and/or regulate the transporter function of the dog PGP, and for determining the portions of the dog PGP which are required for transporter activity.

Variants which are non-functional also can be prepared as described above. Such  
25 variants are useful, for example, as negative controls in experiments testing transporter activity.

A dog PGP nucleic acid, in one embodiment, is operably linked to a gene expression sequence which directs the expression of the dog PGP nucleic acid within a eukaryotic or prokaryotic cell. The “gene expression sequence” is any regulatory nucleotide sequence, such  
30 as a promoter sequence or promoter-enhancer combination, which facilitates the efficient transcription and translation of the dog PGP nucleic acid to which it is operably linked. The gene expression sequence may, for example, be a mammalian or viral promoter, such as a

constitutive or inducible promoter. Constitutive mammalian promoters include, but are not limited to, the promoters for the following genes: hypoxanthine phosphoribosyl transferase (HPTR), adenosine deaminase, pyruvate kinase,  $\beta$ -actin promoter and other constitutive promoters. Exemplary viral promoters which function constitutively in eukaryotic cells include, for example, promoters from the simian virus, papilloma virus, adenovirus, human immunodeficiency virus (HIV), Rous sarcoma virus, cytomegalovirus, the long terminal repeats (LTR) of Moloney murine leukemia virus and other retroviruses, and the thymidine kinase promoter of herpes simplex virus. Other constitutive promoters are known to those of ordinary skill in the art. The promoters useful as gene expression sequences of the invention also include inducible promoters. Inducible promoters are expressed in the presence of an inducing agent. For example, the metallothionein promoter is induced to promote transcription and translation in the presence of certain metal ions. Other inducible promoters are known to those of ordinary skill in the art.

In general, the gene expression sequence shall include, as necessary, 5' non-transcribing and 5' non-translating sequences involved with the initiation of transcription and translation, respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribing sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined dog PGP nucleic acid. The gene expression sequences optionally includes enhancer sequences or upstream activator sequences as desired.

The dog PGP nucleic acid sequence and the gene expression sequence are said to be "operably linked" when they are covalently linked in such a way as to place the transcription and/or translation of the dog PGP coding sequence under the influence or control of the gene expression sequence. If it is desired that the dog PGP sequence be translated into a functional protein, two DNA sequences are said to be operably linked if induction of a promoter in the 5' gene expression sequence results in the transcription of the dog PGP sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the dog PGP sequence, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a gene expression sequence would be operably linked to a dog PGP nucleic acid sequence if the gene expression sequence were capable of effecting transcription of that dog PGP nucleic acid sequence such that the

resulting transcript might be translated into the desired protein or polypeptide.

The dog PGP nucleic acid molecules and the dog PGP polypeptides (including the dog PGP inhibitors described below) of the invention can be delivered to the eukaryotic or prokaryotic cell alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating: (1) delivery of a dog PGP nucleic acid or polypeptide to a target cell, (2) uptake of a dog PGP nucleic acid or polypeptide by a target cell, or (3) expression of a dog PGP nucleic acid molecule or polypeptide in a target cell. Preferably, the vectors transport the dog PGP nucleic acid or polypeptide into the target cell with reduced degradation relative to the extent of degradation that would result in the absence of the vector.

Optionally, a "targeting ligand" can be attached to the vector to selectively deliver the vector to a cell which expresses on its surface the cognate receptor (e.g. a receptor, an antigen recognized by an antibody) for the targeting ligand. In this manner, the vector (containing a dog PGP nucleic acid or a dog PGP polypeptide) can be selectively delivered to a specific cell. In general, the vectors useful in the invention are divided into two classes: biological vectors and chemical/physical vectors. Biological vectors are more useful for delivery/uptake of dog PGP nucleic acids to/by a target cell. Chemical/physical vectors are more useful for delivery/uptake of dog PGP nucleic acids or dog PGP proteins to/by a target cell.

Biological vectors include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the nucleic acid sequences of the invention, and free nucleic acid fragments which can be linked to the nucleic acid sequences of the invention. Viral vectors are a preferred type of biological vector and include, but are not limited to, nucleic acid sequences from the following viruses: retroviruses, such as Moloney murine leukemia virus; Harvey murine sarcoma virus; murine mammary tumor virus; Rous sarcoma virus; adenovirus; adeno-associated virus; SV40-type viruses; polyoma viruses; poxviruses; retroviruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; and polio virus. One can readily employ other vectors not named but known in the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses, the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. In general, the retroviruses are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but

incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes *in vivo*. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell line with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Kriegler, M., "Gene Transfer and Expression, A Laboratory Manual," W.H. Freeman C.O., New York (1990) and Murry, E.J. Ed. "Methods in Molecular Biology," vol. 7, Humana Press, Inc., Clifton, New Jersey (1991).

Another preferred virus for certain applications is the adeno-associated virus, a double-stranded DNA virus. The adeno-associated virus can be engineered to be replication-deficient and is capable of infecting a wide range of cell types and species. It further has advantages, such as heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Expression vectors containing all the necessary elements for expression are commercially available and known to those skilled in the art. See, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, 1989. Cells are genetically engineered by the introduction into the cells of heterologous DNA (RNA) encoding a dog PGP polypeptide or fragment or variant thereof. That heterologous DNA (RNA) is placed under operable control of transcriptional elements to permit the expression of the heterologous DNA in the host cell.

Preferred systems for mRNA expression in mammalian cells are those such as pRc/CMV (available from Invitrogen, Carlsbad, CA) that contain a selectable marker such as a gene that confers G418 resistance (which facilitates the selection of stably transfected cell lines) and the human cytomegalovirus (CMV) enhancer-promoter sequences. Additionally, suitable for expression in primate or canine cell lines is the pCEP4 vector (Invitrogen), which

contains an Epstein Barr virus (EBV) origin of replication, facilitating the maintenance of plasmid as a multicopy extrachromosomal element. Another expression vector is the pEF-BOS plasmid containing the promoter of polypeptide Elongation Factor 1 $\alpha$ , which stimulates efficiently transcription *in vitro*. The plasmid is described by Mishizuma and Nagata (*Nuc. Acids Res.* 18:5322, 1990), and its use in transfection experiments is disclosed by, for example, Demoulin (*Mol. Cell. Biol.* 16:4710-4716, 1996). Still another preferred expression vector is an adenovirus, described by Stratford-Perricaudet, which is defective for E1 and E3 proteins (*J. Clin. Invest.* 90:626-630, 1992).

In addition to the biological vectors, chemical/physical vectors may be used to deliver a dog PGP nucleic acid or polypeptide to a target cell and facilitate uptake thereby. As used herein, a “chemical/physical vector” refers to a natural or synthetic molecule, other than those derived from bacteriological or viral sources, capable of delivering the isolated dog PGP nucleic acid or polypeptide to a cell.

A preferred chemical/physical vector of the invention is a colloidal dispersion system. Colloidal dispersion systems include lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. A preferred colloidal system of the invention is a liposome. Liposomes are artificial membrane vesicles which are useful as a delivery vector *in vivo* or *in vitro*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2 - 4.0  $\mu$  can encapsulate large macromolecules. RNA, DNA, and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, et al., *Trends Biochem. Sci.*, v. 6, p. 77 (1981)). In order for a liposome to be an efficient nucleic acid transfer vector, one or more of the following characteristics should be present: (1) encapsulation of the nucleic acid of interest at high efficiency with retention of biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information.

Liposomes may be targeted to a particular tissue by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein. Ligands which may be useful for targeting a liposome to a particular cell will depend on the particular cell or tissue type. Additionally when the vector encapsulates a nucleic acid, the vector may be coupled to a nuclear targeting peptide, which will direct the dog PGP nucleic acid to the nucleus of the host cell.

Liposomes are commercially available from Gibco BRL, for example, as LIPOFECTIN™ and LIPOFECTACE™, which are formed of cationic lipids such as N-[1-(2, 3 dioleyloxy)-propyl]-N, N, N-trimethylammonium chloride (DOTMA) and dimethyl dioctadecylammonium bromide (DDAB). Methods for making liposomes are well known in  
5 the art and have been described in many publications.

Other exemplary compositions that can be used to facilitate uptake by a target cell of the dog PGP nucleic acids include calcium phosphate and other chemical mediators of intracellular transport, microinjection compositions, electroporation and homologous recombination compositions (e.g., for integrating a dog PGP nucleic acid into a preselected  
10 location within a target cell chromosome).

The invention also embraces so-called expression kits, which allow the artisan to prepare a desired expression vector or vectors. Such expression kits include at least separate portions of the previously discussed coding sequences. Other components may be added, as desired, as long as the previously mentioned sequences, which are required, are included.

It will also be recognized that the invention embraces the use of the dog PGP cDNA sequences in expression vectors, as well as to transfect host cells and cell lines, be these prokaryotic (e.g., *E. coli*), or eukaryotic (e.g., COS cells, yeast expression systems and recombinant baculovirus expression in insect cells). Especially useful are mammalian cells such as human, pig, goat, primate, etc. They may be of a wide variety of tissue types, and include primary cells and cell lines. Specific examples include intestinal cells and liver cells. The expression vectors require that the pertinent sequence, i.e., those nucleic acids described  
20 *supra*, be operably linked to a promoter.

The invention also provides isolated dog PGP polypeptides which include the amino acid sequence of SEQ ID NO:2, and fragments thereof, encoded by the dog PGP nucleic acids  
25 described above. Dog PGP polypeptides also embrace alleles, such as SEQ ID NOS:23, 25 and 27 (except not SEQ ID NOS:3 and 5), functionally equivalent variants and analogs (those non-allelic polypeptides which vary in amino acid sequence from the disclosed dog PGP polypeptides by 1, 2, 3, 4, 5, or more amino acids) provided that such polypeptides retain dog PGP activity. Non-functional variants also are embraced by the invention; these are useful as  
30 antagonists of transporter function, as negative controls in assays, and the like. Such alleles, variants, analogs and fragments are useful, for example, alone or as fusion proteins for a variety of purposes including as a component of assays.

Fragments of a polypeptide preferably are those fragments which retain a distinct functional capability of the dog PGP polypeptide, in particular as a transporter of various molecules. Other functional capabilities which can be retained in a fragment of a dog PGP polypeptide include interaction with antibodies and interaction with other polypeptides (such as would be found in a protein complex). Those skilled in the art are well versed in methods for selecting fragments which retain a functional capability of the dog PGP. Confirmation of the functional capability of the fragment can be carried out by synthesis of the fragment and testing of the capability according to standard methods. For example, to test the transporter activity of a dog PGP fragment, one inserts or expresses the fragment in a cell in which molecular transport can be measured. Such methods, which are standard in the art, are described further herein.

The invention embraces variants of the dog PGP polypeptides described above. As used herein, a “variant” of a dog PGP polypeptide is a polypeptide which contains one or more modifications to the primary amino acid sequence of a dog PGP polypeptide. Modifications which create a dog PGP variant can be made to a dog PGP polypeptide for a variety of reasons, including 1) to reduce or eliminate an activity of a dog PGP polypeptide, such as transport; 2) to enhance a property of a dog PGP polypeptide, such as protein stability in an expression system or the stability of protein-protein binding; 3) to provide a novel activity or property to a dog PGP polypeptide, such as addition of an antigenic epitope or addition of a detectable moiety; or 4) to establish that an amino acid substitution does or does not affect molecular transport activity. Modifications to a dog PGP polypeptide are typically made to the nucleic acid which encodes the dog PGP polypeptide, and can include deletions, point mutations, truncations, amino acid substitutions and additions of amino acids or non-amino acid moieties. Alternatively, modifications can be made directly to the polypeptide, such as by cleavage, addition of a linker molecule, addition of a detectable moiety, such as biotin, addition of a fatty acid, and the like. Modifications also embrace fusion proteins comprising all or part of the dog PGP amino acid sequence. One of skill in the art will be familiar with methods for predicting the effect on protein conformation of a change in protein sequence, and can thus “design” a variant dog PGP according to known methods. One example of such a method is described by Dahiyat and Mayo in *Science* 278:82-87, 1997, whereby proteins can be designed *de novo*. The method can be applied to a known protein to vary only a portion of the polypeptide sequence. By applying the computational methods of

Dahiyat and Mayo, specific variants of a dog PGP polypeptide can be proposed and tested to determine whether the variant retains a desired conformation.

Variants include dog PGP polypeptides which are modified specifically to alter a feature of the polypeptide unrelated to its physiological activity. For example, cysteine residues can be substituted or deleted to prevent unwanted disulfide linkages. Similarly, certain amino acids can be changed to enhance expression of a dog PGP polypeptide by eliminating proteolysis by proteases in an expression system (e.g., dibasic amino acid residues in yeast expression systems in which KEX2 protease activity is present).

Mutations of a nucleic acid which encode a dog PGP polypeptide preferably preserve the amino acid reading frame of the coding sequence, and preferably do not create regions in the nucleic acid which are likely to hybridize to form secondary structures, such as hairpins or loops, which can be deleterious to expression of the variant polypeptide.

Mutations can be made by selecting an amino acid substitution, or by random mutagenesis of a selected site in a nucleic acid which encodes the polypeptide. Variant polypeptides are then expressed and tested for one or more activities to determine which mutation provides a variant polypeptide with a desired property. Further mutations can be made to variants (or to non-variant dog PGP polypeptides) which are silent as to the amino acid sequence of the polypeptide, but which provide preferred codons for translation in a particular host. The preferred codons for translation of a nucleic acid in, e.g., *E. coli*, are well known to those of ordinary skill in the art. Still other mutations can be made to the noncoding sequences of a dog PGP gene or cDNA clone to enhance expression of the polypeptide.

The activity of variants of dog PGP polypeptides can be tested by cloning the gene encoding the variant dog PGP polypeptide into a bacterial or mammalian expression vector, introducing the vector into an appropriate host cell, expressing the variant dog PGP polypeptide, and testing for a functional capability of the dog PGP polypeptides as disclosed herein. For example, the variant dog PGP polypeptide can be tested for ability to provide molecular transport (e.g., efflux), as set forth below in the examples. Preparation of other variant polypeptides may favor testing of other activities, as will be known to one of ordinary skill in the art.

The skilled artisan will also realize that conservative amino acid substitutions may be made in dog PGP polypeptides to provide functionally equivalent variants of the foregoing polypeptides, i.e, variants which retain the functional capabilities of the dog PGP

polypeptides. As used herein, a “conservative amino acid substitution” refers to an amino acid substitution which does not alter the relative charge or size characteristics of the polypeptide in which the amino acid substitution is made. Variants can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as 5 are found in references which compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. Exemplary functionally equivalent variants of the dog PGP polypeptides include conservative amino acid substitutions of SEQ ID NO:2 10 or SEQ ID NO:4. Conservative substitutions of amino acids include substitutions made amongst amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D.

Conservative amino-acid substitutions in the amino acid sequence of dog PGP polypeptide to produce functionally equivalent variants of dog PGP typically are made by alteration of the nucleic acid sequence encoding dog PGP polypeptides (e.g., SEQ ID NO:1). Such substitutions can be made by a variety of methods known to one of ordinary skill in the art. For example, amino acid substitutions may be made by PCR-directed mutation, site-directed mutagenesis according to the method of Kunkel (Kunkel, *Proc. Nat. Acad. Sci. U.S.A.* 82: 488-492, 1985), or by chemical synthesis of a gene encoding a dog PGP 15 polypeptide. The activity of functionally equivalent fragments of dog PGP polypeptides can be tested by cloning the gene encoding the altered dog PGP polypeptide into a bacterial or mammalian expression vector, introducing the vector into an appropriate host cell, expressing the altered dog PGP polypeptide, and testing for the ability of the dog PGP polypeptide to mediate transmembrane transport of compounds. Peptides which are chemically synthesized 20 can be tested directly for function.

A variety of methodologies well-known to the skilled practitioner can be utilized to obtain isolated dog PGP molecules. The polypeptide may be purified from cells which naturally produce the polypeptide by chromatographic means or immunological recognition. Alternatively, an expression vector may be introduced into cells to cause production of the 25 polypeptide. In another method, mRNA transcripts may be microinjected or otherwise introduced into cells to cause production of the encoded polypeptide. Translation of mRNA in cell-free extracts such as the reticulocyte lysate system also may be used to produce

polypeptide. Those skilled in the art also can readily follow known methods for isolating dog PGP polypeptides. These include, but are not limited to, immunochromatography, HPLC, size-exclusion chromatography, ion-exchange chromatography and immune-affinity chromatography.

5 The invention as described herein has a number of uses, some of which are described elsewhere herein. For example, the invention permits isolation of the dog PGP polypeptide molecules by e.g., expression of a recombinant nucleic acid to produce large quantities of polypeptide which may be isolated using standard protocols. As another example, the isolation of the dog PGP gene makes it possible for dog PGP to be used in methods for  
10 assaying of molecular transport, such as drug bioavailability studies. These methods involve determining transport of a drug by a first species' PGP (e.g., dog) in comparison to transport of the drug by other species' PGP (e.g. human) as a method for determining or predicting the bioavailability of the drug. Thus the results of whole animal studies on the metabolism of a drug can be evaluated in view of the relative rates or amounts of P-glycoprotein transport of the drug. For example, if a drug administered to a dog has good oral bioavailability and low transport by dog PGP, one can predict that the oral bioavailability of the drug in humans will be good if the transport by human PGP is also low. Conversely, if the transport of the drug by human PGP is high, then the bioavailability of the drug would be predicted to be low.  
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20 The invention also embraces agents which bind selectively to the dog PGP nucleic acid molecules or polypeptides as well as agents which bind to variants and fragments of the polypeptides and nucleic acids as described herein. The agents include polypeptides which bind to dog PGP, and antisense nucleic acids, both of which are described in greater detail below. The agents can inhibit or increase dog PGP activity (antagonists and agonists, respectively).

25 Some of the agents are inhibitors. A dog PGP inhibitor is an agent that inhibits dog PGP mediated transport of molecules across a cell membrane. Efflux assays can be performed to screen and/or determine whether a dog PGP inhibitor has the ability to inhibit dog PGP activity, and whether the inhibition is selective. An exemplary assay of efflux is described below in the Examples.

30 In one embodiment the dog PGP inhibitor is an antisense oligonucleotide that selectively binds to a dog PGP nucleic acid molecule, to reduce the expression of dog PGP (or other species' PGPs) in a cell. This is desirable in virtually any medical condition wherein a

reduction of PGP transporter activity is desirable, e.g., to increase retention of cytotoxic agents in a cell.

As used herein, the term "antisense oligonucleotide" or "antisense" describes an oligonucleotide that is an oligoribonucleotide, oligodeoxyribonucleotide, modified oligoribonucleotide, or modified oligodeoxyribonucleotide which hybridizes under physiological conditions to DNA comprising a particular gene or to an mRNA transcript of that gene and, thereby, inhibits the transcription of that gene and/or the translation of that mRNA. The antisense molecules are designed so as to interfere with transcription or translation of a target gene upon hybridization with the target gene or transcript. Those skilled in the art will recognize that the exact length of the antisense oligonucleotide and its degree of complementarity with its target will depend upon the specific target selected, including the sequence of the target and the particular bases which comprise that sequence. It is preferred that the antisense oligonucleotide be constructed and arranged so as to bind selectively with the target under physiological conditions, i.e., to hybridize substantially more to the target sequence than to any other sequence in the target cell under physiological conditions. Based upon SEQ ID NO:1, or upon allelic (except SEQ ID NOS:3 and 5) or homologous genomic and/or cDNA sequences, one of skill in the art can easily choose and synthesize any of a number of appropriate antisense molecules for use in accordance with the present invention. In order to be sufficiently selective and potent for inhibition, such antisense oligonucleotides should comprise at least 10 and, more preferably, at least 15 consecutive bases which are complementary to the target, although in certain cases modified oligonucleotides as short as 7 bases in length have been used successfully as antisense oligonucleotides (Wagner et al., *Nature Biotechnol.* 14:840-844, 1996). Most preferably, the antisense oligonucleotides comprise a complementary sequence of 20-30 bases. Although oligonucleotides may be chosen which are antisense to any region of the gene or mRNA transcripts, in preferred embodiments the antisense oligonucleotides correspond to N-terminal or 5' upstream sites such as translation initiation, transcription initiation or promoter sites. In addition, 3'-untranslated regions may be targeted. Targeting to mRNA splicing sites has also been used in the art but may be less preferred if alternative mRNA splicing occurs. In addition, the antisense is targeted, preferably, to sites in which mRNA secondary structure is not expected (see, e.g., Sainio et al., *Cell Mol. Neurobiol.* 14(5):439-457, 1994) and at which polypeptides are not expected to bind. Thus, the present invention also provides for antisense

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oligonucleotides which are complementary to allelic (except SEQ ID NOS:3 and 5) or homologous cDNAs and genomic DNAs corresponding to a dog PGP nucleic acid containing SEQ ID NO:1.

In one set of embodiments, the antisense oligonucleotides of the invention may be  
5 composed of "natural" deoxyribonucleotides, ribonucleotides, or any combination thereof. That is, the 5' end of one native nucleotide and the 3' end of another native nucleotide may be covalently linked, as in natural systems, via a phosphodiester internucleoside linkage. These oligonucleotides may be prepared by art recognized methods which may be carried out manually or by an automated synthesizer. They also may be produced recombinantly by  
10 vectors.

In preferred embodiments, however, the antisense oligonucleotides of the invention also may include "modified" oligonucleotides. That is, the oligonucleotides may be modified in a number of ways which do not prevent them from hybridizing to their target but which enhance their stability or targeting or which otherwise enhance their therapeutic effectiveness.

The term "modified oligonucleotide" as used herein describes an oligonucleotide in which (1) at least two of its nucleotides are covalently linked via a synthetic internucleoside linkage (i.e., a linkage other than a phosphodiester linkage between the 5' end of one nucleotide and the 3' end of another nucleotide) and/or (2) a chemical group not normally associated with nucleic acids has been covalently attached to the oligonucleotide. Preferred synthetic internucleoside linkages are phosphorothioates, alkylphosphonates, phosphorodithioates, phosphate esters, alkylphosphonothioates, phosphoramidates, carbamates, carbonates, phosphate triesters, acetamidates, carboxymethyl esters and peptides.

The term "modified oligonucleotide" also encompasses oligonucleotides with a covalently modified base and/or sugar. For example, modified oligonucleotides include  
25 oligonucleotides having backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified oligonucleotides may include a 2'-O-alkylated ribose group. In addition, modified oligonucleotides may include sugars such as arabinose instead of ribose. The present invention, thus, contemplates pharmaceutical  
30 preparations containing modified antisense molecules that are complementary to and hybridizable with, under physiological conditions, nucleic acids encoding dog PGP polypeptides, together with pharmaceutically acceptable carriers.

Agents which bind dog PGP also include binding peptides and other molecules which bind to the dog PGP polypeptide and complexes containing the dog PGP polypeptide. When the binding molecules are inhibitors, the molecules bind to and inhibit the activity of dog PGP. To determine whether a dog PGP binding agent binds to dog PGP any known binding assay may be employed. For example, the binding agent may be immobilized on a surface and then contacted with a labeled dog PGP polypeptide. The amount of dog PGP which interacts with the dog PGP binding agent or the amount which does not bind to the dog PGP binding agent may then be quantitated to determine whether the dog PGP binding agent binds to dog PGP.

The dog PGP binding agents include molecules of numerous size and type that bind selectively or preferentially to dog PGP polypeptides, and complexes of both dog PGP polypeptides and their binding partners. These molecules may be derived from a variety of sources. For example, dog PGP binding agents can be provided by screening degenerate peptide libraries which can be readily prepared in solution, in immobilized form or as phage display libraries. Combinatorial libraries also can be synthesized of peptides containing one or more amino acids. Libraries further can be synthesized of peptoids and non-peptide synthetic moieties.

Phage display can be particularly effective in identifying binding peptides useful according to the invention. Briefly, one prepares a phage library (using e.g. m13, fd, or lambda phage), displaying inserts from 4 to about 80 amino acid residues using conventional procedures. The inserts may represent, for example, a completely degenerate or biased array. One then can select phage-bearing inserts which bind to the dog PGP polypeptide. This process can be repeated through several cycles of reselection of phage that bind to the dog PGP polypeptide. Repeated rounds lead to enrichment of phage bearing particular sequences.

DNA sequence analysis can be conducted to identify the sequences of the expressed polypeptides. The minimal linear portion of the sequence that binds to the dog PGP polypeptide can be determined. One can repeat the procedure using a biased library containing inserts containing part or all of the minimal linear portion plus one or more additional degenerate residues upstream or downstream thereof. Yeast two-hybrid screening methods also may be used to identify polypeptides that bind to the dog PGP polypeptides. Thus, the dog PGP polypeptides of the invention, or a fragment thereof, can be used to screen peptide libraries, including phage display libraries, to identify and select peptide binding

partners of the dog PGP polypeptides of the invention. Such molecules can be used, as described, for screening assays, for purification protocols, for interfering directly with the functioning of dog PGP and for other purposes that will be apparent to those of ordinary skill in the art.

5 Therefore the invention generally provides efficient methods of identifying pharmacological agents or lead compounds for agents useful in the treatment of conditions associated with aberrant PGP activity and the compounds and agents so identified. Generally, the screening methods involve assaying for compounds which inhibit or enhance transport of molecules through dog PGP. Such methods are adaptable to automated, high throughput 10 screening of compounds. Examples of such methods are described in US patent 5,429,921.

A variety of assays for pharmacological agents are provided, including, labeled *in vitro* protein binding assays, efflux assays using detectable molecules, etc. For example, protein binding screens are used to rapidly examine the binding of candidate pharmacological agents to a dog PGP. The candidate pharmacological agents can be derived from, for example, combinatorial peptide libraries. Convenient reagents for such assays are known in the art. An exemplary cell-based assay of efflux involves contacting a cell having a dog PGP with a candidate pharmacological agent under conditions whereby the efflux of a detectably labeled molecule can occur. Specific conditions are well known in the art and are described, for example, in Sharom et al., *Biochem. Pharmacol.* 58:571-586, 1999, and references cited therein. A reduction in the efflux in the presence of the candidate pharmacological agent indicates that the candidate pharmacological agent reduces the efflux activity of dog PGP. An increase in the efflux in the presence of the candidate pharmacological agent indicates that the candidate pharmacological agent increases the efflux activity of dog PGP.

Dog PGP used in the methods of the invention can be added to an assay mixture as an 25 isolated polypeptide (where binding of a candidate pharmaceutical agent is to be measured) or as a cell or other membrane-encapsulated space which includes a dog PGP polypeptide. In the latter assay configuration, the cell or other membrane-encapsulated space can contain the dog PGP as a preloaded polypeptide or as a nucleic acid (e.g. a cell transfected with an expression vector containing a dog PGP). In the assays described herein, the dog PGP 30 polypeptide can be produced recombinantly, or isolated from biological extracts, but preferably is synthesized *in vitro*. Dog PGP polypeptides encompass chimeric proteins comprising a fusion of a dog PGP polypeptide with another polypeptide, e.g., a polypeptide

capable of providing or enhancing protein-protein binding, or enhancing stability of the dog PGP polypeptide under assay conditions. A polypeptide fused to a dog PGP polypeptide or fragment thereof may also provide means of readily detecting the fusion protein, e.g., by immunological recognition or by fluorescent labeling.

5 The assay mixture also comprises a candidate pharmacological agent. Typically, a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a different response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e., at zero concentration of agent or at a concentration of agent below the limits of assay detection. Candidate agents encompass numerous chemical classes,  
10 although typically they are organic compounds. Preferably, the candidate pharmacological agents are small organic compounds, i.e., those having a molecular weight of more than 50 yet less than about 2500. Candidate agents comprise functional chemical groups necessary for structural interactions with polypeptides, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups and more preferably at least three of the functional chemical groups. The candidate agents can comprise cyclic carbon or heterocyclic structure and/or aromatic or polyaromatic structures substituted with one or more of the above-identified functional groups. Candidate agents also can be biomolecules such as peptides, saccharides, fatty acids, sterols, isoprenoids, purines, pyrimidines, derivatives or structural analogs of the above, or combinations thereof and the like. Where the agent is a nucleic acid, the agent typically is a DNA or RNA molecule, although modified nucleic acids having non-natural bonds or subunits are also contemplated.

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Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including  
25 expression of randomized oligonucleotides, synthetic organic combinatorial libraries, phage display libraries of random peptides, and the like. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural and synthetically produced libraries and compounds can be readily modified through conventional chemical, physical, and biochemical means. Further,  
30 known pharmacological agents may be subjected to directed or random chemical modifications such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs of the agents.

Therefore, a source of candidate agents are libraries of molecules based on known P-glycoprotein inhibitors, in which the structure of the inhibitor is changed at one or more positions of the molecule to contain more or fewer chemical moieties or different chemical moieties. The structural changes made to the molecules in creating the libraries of analog  
5 inhibitors can be directed, random, or a combination of both directed and random substitutions and/or additions. One of ordinary skill in the art in the preparation of combinatorial libraries can readily prepare such libraries based on existing P-glycoprotein inhibitors.

A variety of other reagents also can be included in the mixture. These include  
10 reagents such as salts, buffers, neutral proteins (e.g., albumin), detergents, etc. which may be used to facilitate optimal protein-protein and/or protein-nucleic acid binding. Such a reagent may also reduce non-specific or background interactions of the reaction components. Other reagents that improve the efficiency of the assay such as protease inhibitors, nuclease  
15 inhibitors, antimicrobial agents, and the like may also be used.

The mixture of the foregoing assay materials is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the dog PGP mediates the efflux of a control amount of a compound such as a drug. For determining the binding of a candidate pharmaceutical agent to a dog PGP, the mixture is incubated under conditions which permit binding. The order of addition of components, incubation temperature, time of incubation, and other parameters of the assay may be readily determined. Such experimentation merely involves optimization of the assay parameters, not the fundamental composition of the assay. Incubation temperatures typically are between 4°C and 40°C. Incubation times preferably are minimized to facilitate rapid, high throughput screening, and typically are between 1 minute and 10 hours.

25 After incubation, the level of efflux or the level of specific binding between the dog PGP polypeptide and the candidate pharmaceutical agent is detected by any convenient method available to the user. For cell free binding type assays, a separation step is often used to separate bound from unbound components. The separation step may be accomplished in a variety of ways. Conveniently, at least one of the components is immobilized on a solid  
30 substrate, from which the unbound components may be easily separated. The solid substrate can be made of a wide variety of materials and in a wide variety of shapes, e.g., microtiter plate, microbead, dipstick, resin particle, etc. The substrate preferably is chosen to maximize

signal to noise ratios, primarily to minimize background binding, as well as for ease of separation and cost.

Separation may be effected for example, by removing a bead or dipstick from a reservoir, emptying or diluting a reservoir such as a microtiter plate well, rinsing a bead, 5 particle, chromatographic column or filter with a wash solution or solvent. The separation step preferably includes multiple rinses or washes. For example, when the solid substrate is a microtiter plate, the wells may be washed several times with a washing solution, which typically includes those components of the incubation mixture that do not participate in specific bindings such as salts, buffer, detergent, non-specific protein, etc. Where the solid 10 substrate is a magnetic bead, the beads may be washed one or more times with a washing solution and isolated using a magnet.

Detection may be effected in any convenient way for cell-based assays such as a transmembrane transport assay. The transport of a directly or indirectly detectable product, e.g., a fluorescent molecule such as calcein AM or rhodamine 123, is preferred. For cell free 15 binding assays, one of the components usually comprises, or is coupled to, a detectable label. A wide variety of labels can be used, such as those that provide direct detection (e.g., radioactivity, luminescence, optical or electron density, etc.) or indirect detection (e.g., epitope tag such as the FLAG epitope, enzyme tag such as horseradish peroxidase, etc.). The label may be bound to a dog PGP polypeptide or the candidate pharmacological agent.

20 A variety of methods may be used to detect the label, depending on the nature of the label and other assay components. For example, the label may be detected while bound to the solid substrate or subsequent to separation from the solid substrate. Labels may be directly detected through optical or electron density, radioactive emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, streptavidin-biotin conjugates, 25 etc. Methods for detecting the labels are well known in the art.

The dog PGP binding agent may also be an antibody or a functionally active antibody 30 fragment. Antibodies are well known to those of ordinary skill in the science of immunology. As used herein, the term "antibody" means not only intact antibody molecules but also fragments of antibody molecules retaining dog PGP binding ability. Such fragments are also well known in the art and are regularly employed both *in vitro* and *in vivo*. In particular, as used herein, the term "antibody" means not only intact immunoglobulin molecules but also the well-known active fragments  $F(ab')_2$ , and Fab.  $F(ab')_2$ , and Fab fragments which lack the

Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl et al., *J. Nucl. Med.* 24:316-325 (1983)).

Monoclonal antibodies may be made by any of the methods known in the art utilizing 5 dog PGP, or a fragment thereof, as an immunogen. Alternatively the antibody may be a polyclonal antibody specific for dog PGP which inhibits dog PGP activity. The preparation and use of polyclonal antibodies is also known to one of ordinary skill in the art.

Significantly, as is well known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in 10 general, Clark, W.R. (1986) *The Experimental Foundations of Modern Immunology* Wiley & Sons, Inc., New York; Roitt, I. (1991) *Essential Immunology*, 7th Ed., Blackwell Scientific Publications, Oxford). The pFc' and Fc regions, for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has been enzymatically cleaved, or which has been produced without the pFc' region, designated 15 an F(ab')<sub>2</sub> fragment, retains both of the antigen binding sites of an intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an Fab fragment, retains one of the antigen binding sites of an intact antibody molecule. Proceeding further, Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. 20 The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

Within the antigen-binding portion of an antibody, as is well-known in the art, there are complementarity determining regions (CDRs), which directly interact with the epitope of 25 the antigen, and framework regions (FRs), which maintain the tertiary structure of the paratope (see, in general, Clark, 1986; Roitt, 1991). In both the heavy chain Fd fragment and the light chain of IgG immunoglobulins, there are four framework regions (FR1 through FR4) separated respectively by three complementarity determining regions (CDR1 through CDR3). The CDRs, and in particular the CDR3 regions, and more particularly the heavy chain CDR3, 30 are largely responsible for antibody specificity.

In general, intact antibodies are said to contain "Fc" and "Fab" regions. The Fc regions are involved in complement activation and are not involved in antigen binding. An

antibody from which the Fc' region has been enzymatically cleaved, or which has been produced without the Fc' region, designated an "F(ab')<sub>2</sub>" fragment, retains both of the antigen binding sites of the intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an 5 "Fab" fragment, retains one of the antigen binding sites of the intact antibody. Fab' fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain, denoted "Fd." The Fd fragments are the major determinants of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity). Isolated Fd fragments retain the ability to specifically 10 bind to antigen epitopes.

The sequences of the antigen-binding Fab' portion of the anti-dog PGP monoclonal antibodies identified as being useful according to the invention in the assays provided above, as well as the relevant FR and CDR regions, can be determined using amino acid sequencing methods that are routine in the art. It is well established that non-CDR regions of a 15 mammalian antibody may be replaced with corresponding regions of non-specific or hetero-specific antibodies while retaining the epitope specificity of the original antibody. This technique is useful for the development and use of "humanized" antibodies in which non-human CDRs are covalently joined to human FR and/or Fc/pFc' regions to produce a functional antibody. Techniques to humanize antibodies are particularly useful when non-human animal (e.g., murine) antibodies which inhibit dog PGP activity are identified. These 20 non-human animal antibodies can be humanized for use in the treatment of a human subject in the methods according to the invention. Examples of methods for humanizing a murine antibody are provided in U.S. patents 4,816,567, 5,225,539, 5,585,089, 5,693,762 and 5,859,205. Other antibodies, including fragments of intact antibodies with antigen-binding 25 ability, are often referred to as "chimeric" antibodies.

Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for F(ab')<sub>2</sub>, and Fab fragments of an anti-dog PGP monoclonal antibody; chimeric antibodies in which the Fc and/or FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions of an anti-dog PGP antibody have been replaced by homologous human or non-human sequences; chimeric F(ab')<sub>2</sub> fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions of an anti-dog PGP antibody have been replaced by homologous human or non-human sequences; and chimeric Fab fragment antibodies in which

the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences.

According to the invention dog PGP inhibitors also include “dominant negative” polypeptides derived from SEQ ID NOs:2. A dominant negative polypeptide is an inactive 5 variant of a polypeptide, which, by interacting with the cellular machinery, displaces an active polypeptide from its interaction with the cellular machinery or competes with the active polypeptide, thereby reducing the effect of the active polypeptide. For example, a dominant negative receptor which binds a ligand but does not transmit a signal in response to binding of the ligand can reduce the biological effect of expression of the ligand.

10 The end result of the expression of a dominant negative dog PGP polypeptide of the invention in a cell is a reduction in PGP activity such as molecular transport. One of ordinary skill in the art can assess the potential for a dominant negative variant of a dog PGP polypeptide, and using standard mutagenesis techniques to create one or more dominant negative variant polypeptides. For example, given the teachings contained herein of a dog PGP polypeptide, one of ordinary skill in the art can modify the sequence of the dog PGP polypeptide by site-specific mutagenesis, scanning mutagenesis, partial gene deletion or truncation, and the like. See, e.g., U.S. Patent No. 5,580,723 and Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, 1989. 15 The skilled artisan then can test the population of mutagenized polypeptides for diminution in dog PGP activity and/or for retention of such an activity. Other similar methods for creating and testing dominant negative variants of a dog PGP polypeptide will be apparent to one of ordinary skill in the art. 20

25 Each of the compositions of the invention is useful for a variety of therapeutic and non-therapeutic purposes. For example, the dog PGP nucleic acids of the invention are useful as oligonucleotide probes. Such oligonucleotide probes can be used herein to identify genomic or cDNA library clones possessing an identical or substantially similar nucleic acid sequence. A suitable oligonucleotide or set of oligonucleotides, which is capable of hybridizing under stringent hybridization conditions to the desired sequence, a variant or fragment thereof, or an anti-sense complement of such an oligonucleotide or set of 30 oligonucleotides, can be synthesized by means well known in the art (see, for example, *Synthesis and Application of DNA and RNA*, S.A. Narang, ed., 1987, Academic Press, San Diego, CA) and employed as a probe to identify and isolate the desired sequence, variant or

fragment thereof by techniques known in the art. Techniques of nucleic acid hybridization and clone identification are disclosed by Sambrook, et al., *Molecular Cloning, A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, Plainview, NY (1989). To facilitate the detection of a desired nucleic acid sequence, or variant or fragment thereof, whether for 5 cloning purposes or for the mere detection of the presence of the sequence, the above-described probes may be labeled with a detectable group. Such a detectable group may be any material having a detectable physical or chemical property. Such materials have been well-developed in the field of nucleic acid hybridization and, in general, many labels useful in such methods can be applied to the present invention. Particularly useful are radioactive 10 labels. Any radioactive label may be employed which provides for an adequate signal and has a sufficient half-life. If single stranded, the oligonucleotide may be radioactively labeled using kinase reactions. Alternatively, oligonucleotides are also useful as nucleic acid hybridization probes when labeled with a non-radioactive marker such as biotin, an enzyme or a fluorescent group. See, for example, Leary, J.J., et al., *Proc. Natl. Acad. Sci. (USA)* 80:4045 15 (1983); Renz, M. et al., *Nucl. Acids Res.* 12:3435 (1984); and Renz, M., *EMBO J.* 6:817 (1983).

20 Additionally, complements of the dog PGP nucleic acids can be useful as antisense oligonucleotides, e.g., by delivering the antisense oligonucleotide to an animal to induce a dog PGP "knockout" phenotype. The administration of antisense RNA probes to block gene expression is discussed in Lichtenstein, C., *Nature* 333:801-802 (1988).

25 Alternatively, the dog PGP nucleic acid of the invention can be used to prepare a non-human transgenic animal. A "transgenic animal" is an animal having cells that contain DNA which has been artificially inserted into a cell, which DNA becomes part of the genome of the animal which develops from that cell. Preferred transgenic animals are primates, mice, rats, cows, pigs, horses, goats, sheep, dogs and cats. Animals suitable for transgenic experiments can be obtained from standard commercial sources such as Charles River (Wilmington, MA), Taconic (Germantown, NY), Harlan Sprague Dawley (Indianapolis, IN), etc. Transgenic animals having a particular property associated with a particular disease can be used to study the affects of a variety of drugs and treatment methods on the disease, and thus serve as 30 genetic models for the study of a number of human diseases. The invention, therefore, contemplates the use of dog PGP knockout and transgenic animals as models for the study of disorders involving transport of molecules across cell membranes. A variety of methods

known to one of ordinary skill in the art are available for the production of transgenic animals associated with this invention.

Inactivation or replacement of the endogenous PGP/MDR1 gene can be achieved by a homologous recombination system using embryonic stem cells. The resultant transgenic non-

5 human mammals having a PGP<sup>-/-</sup> knockout phenotype may be made transgenic for the dog PGP and used as a model for screening compounds as modulators (agonists or antagonists/inhibitors) of the dog PGP. In this manner, such therapeutic drugs can be identified.

10 Additionally, a normal or mutant version of dog PGP can be inserted into the germ line to produce transgenic animals which constitutively or inducibly express the normal or mutant form of dog PGP. These animals are useful in studies to define the role and function of dog PGP in cells.

The compositions of the invention are also useful for therapeutic purposes.

15 Accordingly the invention encompasses a method for inhibiting dog PGP activity in a mammalian cell. The invention further provides methods for reducing or increasing dog PGP activity in a cell. In one embodiment, the method involves contacting the mammalian cell with an amount of a dog PGP nucleic acid or polypeptide effective to inhibit molecular transport out of the mammalian cell. Such methods are useful *in vitro* for the purpose of, for example, elucidating the mechanisms involved in drug resistance and reduced drug 20 bioavailability.

The invention also encompasses a method for increasing PGP expression in a cell or subject. The amount of dog PGP can be increased in such cell or subject by contacting the cell with, or administering to the subject, a PGP nucleic acid or a PGP polypeptide of the invention to the subject in an amount effective to increase transmembrane transport in the cell 25 or the subject. An increase in PGP activity can be measured by the assays described herein, e.g., assays of transmembrane transport.

The preparations of the invention are administered in effective amounts. An effective amount is that amount of a pharmaceutical preparation that alone, or together with further doses, produces the desired response. Such amounts will depend, of course, on the particular 30 condition being treated, the severity of the condition, the individual patient parameters including age, physical condition, size and weight, the duration of the treatment, the nature of concurrent therapy (if any), the specific route of administration and like factors within the

knowledge and expertise of the health practitioner. It is preferred generally that a maximum dose be used, that is, the highest safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a patient may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or for virtually any 5 other reasons.

Generally, doses of active compounds would be from about 0.01 mg/kg per day to 1000 mg/kg per day. It is expected that doses ranging from 50-500 mg/kg will be suitable and in one or several administrations per day. Lower doses will result from other forms of administration, such as intravenous administration. In the event that a response in a subject is 10 insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits. Multiple doses per day are contemplated to achieve appropriate systemic levels of compound, although fewer doses typically will be given when compounds are prepared as slow release or sustained release medications.

When administered, the pharmaceutical preparations of the invention are applied in pharmaceutically-acceptable amounts and in pharmaceutically-acceptably compositions. Such preparations may routinely contain salts, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope 15 of the invention. Such pharmacologically and pharmaceutically-acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic, and the like. Also, pharmaceutically-acceptable salts can be prepared as alkaline metal or alkaline 20 earth salts, such as sodium, potassium or calcium salts.

The dog PGP inhibitors or dog PGP nucleic acids and polypeptides useful according to the invention may be combined, optionally, with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for 25 administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being co-mingled

with the molecules of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

The pharmaceutical compositions may contain suitable buffering agents, including: acetic acid in a salt; citric acid in a salt; and phosphoric acid in a salt.

5 The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as: benzalkonium chloride; chlorobutanol; parabens and thimerosal.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular compound selected, the severity of the condition being treated and the dosage required for therapeutic efficacy. The methods of the invention, 10 generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of the active compounds without causing clinically unacceptable adverse effects. Such modes of administration include oral, rectal, topical, nasal, intradermal, or parenteral routes. The term "parenteral" includes subcutaneous, intravenous, intrathecal, intramuscular, or infusion. Intravenous or 15 intramuscular routes are not particularly suitable for long-term therapy and prophylaxis.

The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly 20 and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active compound. Other compositions include suspensions in aqueous liquids or non-aqueous 25 liquids such as a syrup, elixir or an emulsion.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the dog PGP inhibitor or dog PGP nucleic acids and polypeptides, which is preferably isotonic with the blood of the recipient. This aqueous preparation may be formulated according to known methods using suitable dispersing or wetting agents and 30 suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be

employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier 5 formulation suitable for oral, subcutaneous, intravenous, intrathecal, intramuscular, etc. administrations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA.

Other delivery systems can include time-release, delayed release or sustained release delivery systems such as the biological/chemical vectors is discussed above. Such systems 10 can avoid repeated administrations of the active compound, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. Use of a long-term sustained release implant may be desirable. Long-term release, are used herein, means that the implant is constructed and arranged to delivery therapeutic levels of the active ingredient for at least 30 days, and 15 preferably 60 days. Long-term sustained release implants are well-known to those of ordinary skill in the art and include some of the release systems described above.

The invention will be more fully understood by reference to the following examples. These examples, however, are merely intended to illustrate the embodiments of the invention and are not to be construed to limit the scope of the invention.

20

### Examples

#### **Example 1: Isolation of dog P-glycoprotein**

cDNA libraries were prepared using dog (*Canis familiaris*) mRNA according to 25 standard procedures. The libraries were screened for P-glycoprotein clones using a human P-glycoprotein DNA probe. Clones were isolated, purified and sequenced in accordance with standard procedures, as described below.

Liver and lung tissue was excised from Beagle dog tissue samples and flash frozen in liquid nitrogen.

30 A 890bp fragment of dog PGP1 was PCRed out of a lambda gt11 vector cDNA library custom made by Stratagene. PCR was done using reagents from Clontech. Primers were custom designed, one specific for sequence homologous to human PGP1, the other

homologous to dog PGP2.

<u>Primer name</u>	<u>sequence</u>	<u>size</u>	<u>SEQ NO:</u>	<u>homology</u>
Dp1216F	5'-gaa ctg tga ttg cgt ttg gag gac-3'	24mer	9	human PGP1
Dp2133R	5'-ttc agg gcc gcc tgt acc tct g-3'	22mer	10	dog PGP2

5 PCR was performed with a Perkin Elmer 9700 thermocycler. PCR was done at 94°C for 2 minutes, followed by 40 cycles of 94°C for 30 seconds, 40°C for 20 seconds and 72°C for 2 minutes and 30 seconds, and then incubated at 72°C for 3 minutes. The PCR product was run on a 1% agarose gel, and stained with EtBr. The DNA band at 917bp was cut out, and purified using the QIAquick Gel Extraction kit from Qiagen Inc.

10 The DNA was then ligated to the vector pCR2.1 using the Original TA Cloning kit from Invitrogen. INValphaF' cells were transformed with the ligation retain, a colony was selected, grown up in 100mL of L broth with ampicillin. DNA purification was done using the Plasmid Midi purification kit from Qiagen. The insert was sequenced using an ABI 377 sequencer. This sequence corresponds to an 890bp fragment of dog PGP, from base 816 to base 1706 in the open reading frame.

15 Five other fragments were isolated, cloned and sequenced using the same method as stated above, with any differences in procedure stated below.

20 RNA extraction from dog lung tissue was performed using Nucliobond RNA Maxi Prep. purification kit from Clontech. cDNA was synthesized from the RNA preparation using Superscript Preamplification System for First Strand cDNA Synthesis from Life Technologies.

Fragment 1: cDNA source-dog lung cDNA library prep.

<u>Primer name</u>	<u>sequence</u>	<u>size</u>	<u>SEQ NO:</u>	<u>homology</u>
Dm1766F	5'-ccc cac aga tgg cat ggt ctg t-3'	22mer	11	dog PGP1
Dp2769R	5'-cgc ttg gtg agg atc tct cca gc-3'	23mer	12	human PGP1

25 This corresponds to a 981bp fragment of dog PGP from base 1364 to base 2345 in the open reading frame.

30 Fragment 2: cDNA source dog - lung cDNA library prep.

<u>Primer name</u>	<u>sequence</u>	<u>size</u>	<u>SEQ NO:</u>	<u>homology</u>
Dm2037F	5'-aga aac aga gaa tcg cca ttg ctc-3'	24mer	13	dog PGP1

Dp3793R 5'-gct gca gtc aaa cag gat ggg ct-3' 23mer 14 human PGP1

This corresponds to a 1728bp fragment of dog PGP from base 1635 to base 3362 in the open reading frame.

5 Fragment 3: cDNA source dog - lung cDNA prep

<u>Primer name</u>	<u>sequence</u>	<u>size</u>	<u>SEQ NO:</u>	<u>homology</u>
Dm3411F	5'-agt tca ttt gct cct gac tat gcc-3'	24mer	15	dog PGP1
Dp4214R	5'-gat gcc ttt ctg ggc cag cag c-3'	22mer	16	human PGP1

This corresponds to a 779bp fragment of dog PGP from base 3004 to base 3783 in the open  
10 reading frame.

An RNA preparation from dog liver tissue was performed using the same method as used previously. cDNA was made from it using the SMART RACE cDNA Amplification Kit from Clontech using SMART II oligonucleotide. PCR conditions were changed to 94°C for 5 minutes, followed by 5 cycles of 94°C for 30 seconds and 72°C for 2 minutes. Five cycles of 15 94°C for 30 seconds, 70°C for 45 seconds and 72°C for 2 minutes were performed next. The final 30 cycles were at 94°C for 30 seconds, 68°C for 45 seconds and 72°C for 2 minutes. The PCR was concluded with 72°C for 7 minutes.

20 Fragment 4: cDNA source - dog liver cDNA (SMART RACE) prep.

<u>Primer name</u>	<u>sequence</u>	<u>size</u>	<u>SEQ NO:</u>	<u>homology</u>
Dm3612FL	5'-gag gtg aag aag ggc cag acg ctg gcc ctc-3'	30mer	17	dog PGP1
RACE	5'-cta ata cga ctc act ata ggg caa gca gtg gta aca acg cag agt -3'	18		vector

This corresponds to a 3'end fragment of dog PGP from base 3212 to base 4264 in the open  
25 reading frame.

An RNA preparation from dog liver tissue was performed using the same method as used previously. cDNA was made from it using the SMART RACE cDNA Amplification Kit from Clontech using the gene specific Primer DM1680RL 5'-cgc agc cac tgt tcc caa cca gcg 30 cca ct-3', 29mer, SEQ ID NO:19, dog PGP1.

Fragment 5: cDNA source - dog liver cDNA (SMART RACE with internal primer) prep.

<u>Primer name</u>	<u>sequence</u>	<u>size</u>	<u>SEQ NO:</u>	<u>homology</u>
Dp409F	5'-gga gcg cga ggt cgg gat gga tc- 3'	23mer	20	human PGP1
Dm1355RL	5'-gga gag gac caa gga ggt ccc ata cca gaa a-3' 31mer	21		dog PGP1

This corresponds to an ATG fragment of dog PGP from base -15 to base 945 in the open

5 reading frame.

### Assembly of Clones

1Kb fragment (890bp) and 981bp fragment

DNA preps were made of each fragment. Both fragments were cut with NcoI  
10 restriction enzyme. The 1Kb fragment was also cut with EcoRI. The 981bp fragment was  
also cut with BamHI. Each fragment was purified, ligated together, then the ends were cut, the  
cut molecule was gel purified and ligated into pUC19 cut with EcoRI and BamHI.

15 1Kb fragment (890bp)/981bp fragment and 1728bp fragment

These fragments were assembled as above except that both fragments were cut with  
BamHI and the 1728bp fragment was also cut with XbaI.

20 1Kb fragment (890bp)/981bp fragment/1728bp fragment and ATG fragment

3'end and 779bp fragment

1Kb fragment (890bp)/981bp fragment/1728bp fragment ATG fragment and  
25 3'end/779bp fragment

All of these fragments were assembled as above except that, in some cases, the  
fragments were cut with different restriction enzymes according to their nucleotide sequences.

25 The nucleotide sequence of a dog P-glycoprotein is presented as SEQ ID NO:1. The  
coding sequence consists of nucleotides 17-3859, producing a polypeptide of 1281 amino  
acids (SEQ ID NO:2).

### Example 2: Activity of dog P-glycoprotein

#### 30 *Materials and Methods*

Dog PGP cDNA (SEQ ID NO:1) is introduced into a clonal population of LLC-PK1  
cells in a vector that confers resistance to hygromycin B. LLC-PK1 cells are obtained from

the American Type Culture Collections and are propagated in Medium 199 supplemented to 7% with fetal bovine serum. LLC-PK1 cells are recloned prior to transfection in order to assure homogeneity of the cell population. Briefly, dog PGP cDNA is incorporated into the p222CMV vector. This vector is derived from the p220.2 episomal vector system based on the OriP sequences for Epstein Barr virus and the EBNA-1 gene product (Sugden et al., *Mol. Cell Biol.* 5:410-413, 1985; Yates et al., *Nature (Lond.)* 313: 812-815, 1985). The PGP cDNA is under the control of the cytomegalovirus (CMV) immediate early promoter. The vector confers resistance to hygromycin B. Cells (in 0.4 mL) and DNA (10 to 20  $\mu$ g) were transfected by electroporation using a BTX Electro cell manipulator model 600 using a 2 mm gap cell, 100V, 2500  $\mu$ F capacitance and 72 ohm resistance. After electroporation, the cells are plated in multiwell plates (48 well, Corning Costar) at 10% of confluence. One to two days after transfection hygromycin B is introduced at a final concentration of 400 to 600  $\mu$ g/ml. Cells are refed every 2 to 4 days and are propagated in 400 to 600  $\mu$ g/ml hygromycin B for 6 to 8 days at which point the bulk of the wild type cells are detached. The hygromycin B is reduced to 100  $\mu$ g/ml and maintained in this concentration of hygromycin B. After 14 to 18 days the wells are inspected and wells containing single colonies are trypsinized and scaled up to bulk cultures. Expression of PGP is measured by the polarization of vinblastine (0.1  $\mu$ M) transport in Transwells<sup>TM</sup>.

LLC-PK1 cell based transport studies are conducted in 24 well Transwells<sup>TM</sup> (Corning Costar, Catalog number 3415). Transwells<sup>TM</sup> are prepared by the addition of 0.6 mL media to the basolateral space and 0.1 mL media to the apical space. Cells are seeded at  $4 \times 10^4$  cells per insert (typically in 0.05 mL to 0.15 mL), refed with fresh media every 2 to 4 days and used for transport studies 4 to 8 days post seeding. Transport assays are conducted in Hank's balanced saline (HBSS) buffered with 10 mM HEPES (pH 7 to 7.2). Cell monolayers are rinsed with HBSS prior to use in transport assays. Transport is measured under sink conditions in both the apical to basolateral (A to B) and basolateral to apical (B to A) directions. At least duplicate monolayers are used per determination. At the desired time points, samples are withdrawn from the receiver chamber (apical or basolateral chambers). Quantitation of the amount of compound transported is by liquid scintillation counting (vinblastine) or HPLC with UV or mass spectrometric detection.

Dog PGP cDNA is expressed in insect cells using a baculovirus vector. Membranes are prepared according to the method of (Sarkadi et al., *J. Biol. Chem.* 267: 4854-4858, 1992)

and stored at -80°C until use. ATPase assays are conducted in 96 well microtiter plates. The assays are conducted using a modification of the methods of (Sarkadi et al., 1992 and Druekes et al., *Anal. Biochem.* 230: 173-177, 1995).

A detailed method for each well of a 96 well plate is contained below:

5 A 0.06 ml reaction mixture containing 40 µg membranes, 20 µM Verapamil (positive control) or test drug, and 3-5 mM MgATP, in buffer containing 50 mM Tris-MES, 2 mM EGTA, 50 mM KCl, 2 mM dithiothreitol, and 5 mM sodium azide, is incubated at 37°C for 20 min. An identical reaction mixture containing 100 µM sodium orthovanadate is assayed in parallel.

10 Orthovanadate inhibits PGP by trapping MgADP in the nucleotide binding site. Thus, ATPase activity measured in the presence of orthovanadate represents non-PGP ATPase activity and can be subtracted from the activity generated without orthovanadate to yield vanadate-sensitive ATPase activity. The reaction is stopped by the addition of 30 µl of 10 % SDS + Antifoam A. Two additional reaction mixtures (+ and - orthovanadate) but without MgATP, are also prepared and incubated with the others, and then supplemented with SDS and MgATP, to represent time = 0 min of reaction. The incubations are followed with addition of 200 µl of 35 mM ammonium molybdate in 15 mM zinc acetate:10 % ascorbic acid (1:4) and incubated for an additional 20 min at 37°C. The liberation of inorganic phosphate is detected by its absorbance at 800 nm and quantitated by comparing the absorbance to a phosphate standard curve.

20 Ligand binding assays and assays for measuring inhibition of fluorescent dye uptake are preformed as described by Sharom et al. (*Biochem. Pharmacol.* 58:571-586, 1999).

#### *I. Stable PGP Expression in LLC-PK1 Cells.*

Functional expression of dog PGP is measured by the polarization of transport of 25 vinblastine. Control cells typically demonstrate a B to A / A to B ratio of between 1 and 3. PGP transfected cells demonstrate a much higher ratio. The expression of cDNA-derived dog is stable.

#### *II. Activation of ATPase Activity in PGP Membranes.*

30 The stimulation of ATPase assay provides a rapid measure of the concentration dependence of any interaction of a drug with PGP. The liberated inorganic phosphate is measured by a simple spectrophotometric assay performed in a microtiter plate format. The

testing of multiple drug concentrations allows estimation of the affinity of the drug for PGP and whether saturation of the response was observed.

### *III. Drug Transport Across Cell Monolayers.*

5 The ATPase assay does not directly measure drug transport. In order to examine the concordance between activation of ATPase and actual transport, the rates of transport of the drugs are measured in control LLC-PK1 and dog PGP cell monolayers. For each drug concentration, four measurements are made:

	A:	A to B	Control cells
10	B:	B to A	Control cells
	C:	A to B	PGP cells
	D:	B to A	PGP cells

15 The polarization of transport is calculated in control cells (B/A) and PGP cells (D/C). The intrinsic activity (IA) of PGP is calculated as the sum of the amount PGP facilitated B to A transport in PGP cells relative to control cells (D minus B) and the amount that PGP 20 impeded A to B transport in PGP cells relative to control cells (A minus C). The intrinsic clearance of PGP is calculated from a plot of the concentration dependence data by either calculating the slope of the line under non-saturating conditions or from the calculated apparent Km and Vmax values when saturation is observed. Intrinsic clearance is expressed as mL/m<sup>2</sup>/min.

25 The ATPase data provides useful concentration response data. For example, the apparent Km values for some compounds are in good agreement between the ATPase and transport systems. However, other drugs activate ATPase activity but transport by PGP is not detectable. At the least, ATPase assay can identify a concentration range below which the response to transport by PGP was linear with respect to drug concentration. This should allow simplification of the experimental design for measuring the intrinsic clearance of PGP, an important consideration if large numbers of compounds are to be tested.

### *IV. Bioavailability*

30 Bioavailability studies are performed by performing one or more of the assays described above with two or more different PGP types. The different PGP types can be by different species (e.g., dog and human, cynomologous monkey and human, dog and

cynomologous monkey, etc.) or can be different alleles of the same species. The results of these assays are compared to determine or estimate the bioavailability of a drug in individuals of the different species or in individuals that express different PGP alleles. The results of one determination also may be compared to a previously determined value of, e.g., ATPase or  
5 transport, as an historical control.

*Protocols and Procedures*

Isolation of RNA

Frozen beagle liver tissue was ground up using a mortar and pestle. Pulverized tissue  
10 (300 mg) was transferred to a dounce homogenizer. TRIsol Reagent (3mL; Cat. # 15596 from Life Technologies) was added and the tissue was homogenized then incubated for 15 minutes at room temperature. Aliquots (1mL) of the solution were transferred to three microfuge tubes and 200  $\mu$ L of chloroform were added to each tube. Each tube was vortexed at a high setting for 15 seconds, then incubated at room temperature for 3 minutes before being  
15 centrifuged at 10 000 g for 15 minutes at 4°C. The upper "aqueous" phase was transferred to a new microfuge tube while the remaining lower and inter phases were discarded. 500  $\mu$ L of Isopropanol was added to each of the new tubes and mixed by inverting the tube several times. Each tube was then incubated at room temperature for 10 minutes before being  
20 centrifuged at 10 000 g for 10 more minutes at 4°C. A small white pellet was observed and the supernatant was discarded. 1mL of 75% ethanol was added to each tube. The tubes were vortexed briefly then centrifuged at 10 000 g for 10 minutes at 4°C. The supernatant was discarded and the pellet dried for 5 minutes at room temperature. 50  $\mu$ L of DEPC treated sterile deionized water was added to each tube. Each pellet was resuspended and the contents of each tube were pooled.

25 Optical density of the resuspended pellet was analyzed using a Hitachi U3010 spectrophotometer to determine the quantity of RNA that was isolated. The RNA was then diluted using DEPC treated sterile deionized water to a concentration of 0.55  $\mu$ g/ $\mu$ L. 2 mL of this RNA was split evenly into 2 microfuge tubes. Next, 100  $\mu$ L of 5 M NaCl was added to each tube. Both tubes were heated to 65°C for 15 minutes, then put on ice for 5 minutes. 500  
30  $\mu$ L of Oligo (dT) Cellulose Suspension from the MessageMaker mRNA Isolation System (Cat. # 10551-018 from Life Technologies) were added to both tubes. The tubes were then incubated at 37°C for 10 minutes. The contents of the tubes were transferred to the Filter

Syringe provided with the MessageMaker mRNA Isolation System. After expelling the liquid contents, the pellet in the syringe was washed using Wash Buffer 1 and Wash Buffer 2 according to the manufacturer's instructions. The RNA pellet in the syringe was resuspended in 1 mL of DEPC treated sterile deionized water (65°C), followed by the addition of 2.5  $\mu$ L of 50  $\mu$ g/mL glycogen and 100  $\mu$ L of 7.5M ammonium acetate and mixing by inversion. The 5 contents of the tube were then transferred to a two microfuge tubes and 1 mL of ethanol was added to each tube. The contents of both tubes were mixed by inversion, followed by incubation at -20°C overnight.

The tubes were centrifuged at 10 000 g for 30 minutes at 4°C. The supernatant was 10 discarded and 200  $\mu$ L of 75% ethanol was added to each tube. The tubes were vortexed for 10 seconds followed by centrifugation at 10 000 g for 15 minutes at 4°C. The supernatant was discarded and all remaining liquid was carefully pipetted off. The pellet was resuspended in 10  $\mu$ L of DEPC treated sterile deionized water.

15 Preparation of cDNA

CDNA was prepared using the Marathon cDNA Amplification Kit (Cat. # K1802-1 from Clontech Laboratories, Inc., Palo Alto, CA) as follows. Four microliters of the resuspended mRNA were transferred to a new microfuge tube, and 1  $\mu$ L of one of the following primers at 10  $\mu$ M concentration was added: Dm1680RL cgc agc cac tgt tcc caa cca 20 gcg cca ct (SEQ ID NO:19; custom designed, synthesized by Operon Technologies, Inc.) or cDNA Synthesis Primer, nnt ttt ttt ttt ttt ttt ttt ttt ttc gcc ggc gac tta aga tct t (SEQ ID NO:28), from the Marathon cDNA Amplification Kit. Tubes were incubated at 70°C for 2 minutes then placed on ice for 5 minutes. 2  $\mu$ L of 5x First-Strand Buffer, 1  $\mu$ L of 10mM dNTP Mix and 1  $\mu$ L of 20 U/ $\mu$ L AMV Reverse Transcriptase were added to each tube and 25 gently mixed. The tubes were incubated at 42°C for 1 hour, then placed on ice. 2  $\mu$ L of each single stranded cDNA were used as template in PCR (see below, "PCR conditions"). 48.4  $\mu$ L of DEPC treated deionized water, 16  $\mu$ L of 5x Second-Strand Buffer, 1.6  $\mu$ L of 10mM dNTP Mix and 4  $\mu$ L of 20x Second-Strand Enzyme Cocktail were added to the remaining cDNA sample. The tube was incubated at 16°C for 1.5 hours. 2uL of 5U/uL T4 DNA Polymerase 30 was added to the tube, which was then incubated at 16°C for an additional 45 minutes. 20x EDTA/Glycogen Mix (4  $\mu$ L) was added followed by 430  $\mu$ L of Buffer PN from the QIAquick Nucleotide Removal Kit (Cat. # 28304 from QIAGEN Inc., Valencia, CA). The sample was

then loaded into a QIAquick Spin Column and centrifuged at 10 000 g for 1 minute. The collection tube was emptied and 750  $\mu$ L of PE Buffer (QIAGEN) were added to the QIAquick Spin Column which was again centrifuged at 10 000 g for 1 minute. The collection tube was once again emptied and the QIAquick Spin Column was centrifuged at 10 000 g for 1 minute  
5 to dry the column membrane. 50  $\mu$ L of sterile deionized water heated to 50°C was added to the QIAquick Spin Column and incubated at room temperature for 1 minute before being centrifuged at 10 000 g for 1 minute into a clean microfuge tube.

To precipitate nucleic acid, 10.8  $\mu$ L 7.5M ammonium acetate and 144  $\mu$ L ethanol were added to the tube before it was centrifuged at 10 000 g for 20 minutes. The supernatant was  
10 removed, then 300  $\mu$ L of 75% ethanol was added. The tube was vortexed and centrifuged at 10 000 g for 20 minutes. The supernatant was removed and the sample was resuspended in 12  $\mu$ L of sterile deionized water. 5  $\mu$ L of this cDNA were placed in a clean microfuge tube. 2  $\mu$ L of 10  $\mu$ M Marathon cDNA Adapter, 2  $\mu$ L 5x DNA Ligation Buffer and 1  $\mu$ L of 400U/ $\mu$ L  
15 T4 DNA Ligase, from the Marathon cDNA Amplification Kit, were added to the cDNA and mixed gently. This mixture was incubated over night at 16°C. The ligase was heat inactivated at 70°C for 5 minutes. PCR was done using this sample as a template.

#### PCR conditions

PCR was performed using reagents supplied with the Clontech Advantage 2 kit  
20 (Clontech, palo Alto, CA). Briefly, 2  $\mu$ L of cDNA was placed in a 200  $\mu$ L MicroAmp reaction tube (Cat. # N801-0540, PE Biosystems), then 34  $\mu$ L of sterile deionized water, 5  $\mu$ L of 10x Clontech Advantage 2 Polymerase Mix (Cat. # 4700-1 Clontech Laboratories, Inc.), 3  $\mu$ L of 10 mM Advantage UltraPure PCR Deoxynucleotide Mix (Cat. # 8430-1 Clontech Laboratories, Inc.) and 2  $\mu$ L each of a forward and reverse primer at 10mM concentration  
25 were added. Primers were either Marathon cDNA Adapter Primer 1, cca tcc taa tac gac tca ctg tag ggc (SEQ ID NO:29), from the Marathon cDNA Amplification Kit or various custom gene specific primers made by Operon Technologies Inc. Custom primers include (all listed as 5'-3'): Dm409FL: gga gcg cga ggt cgg gat gga tc (SEQ ID NO:20), Dm1455RL: gca aat gct tca atg ctt ggg gat gcc tgt cca a (SEQ ID NO:30), Dm2037F: aga aac aga gaa tcg cca ttg  
30 ctc (SEQ ID NO:13), Dm3920RL: gag ctg ggt tcc ttt gtc tcc tac tct ggt gtt (SEQ ID NO:31), Dp1216F: gaa ctg tga ttg cgt ttg gag gac (SEQ ID NO:9), and Dp2587R: gca aat gct ggt tgc agg cct cc (SEQ ID NO:32).

Thermocycling was done using a Perkin Elmer 9700 at 94°C for 30 seconds followed by 2 cycles of 94°C for 5 seconds and 72°C for 4 minutes, followed by 16 cycles of 94°C for 5 seconds and 68°C for 4 minutes.

Four fragments were acquired by PCR for each Genotype. Fragment 1 was generated  
5 using primers Marathon cDNA Adapter Primer 1 and Dm1455RL. Fragment 2 was generated using primers Dp1216F and Dp2587R. Fragment 3 was generated using primers Dm2037F and Dm3920RL. Fragment 4 was generated using primers Dp3612FL and Marathon cDNA Adapter Primer 1.

10 Post PCR sample processing

Electrophoresis was performed on all PCR products using 10x Gel-loading Buffer IV (*Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, page 6.12), on a 1% agarose gel in 0.5x TBE buffer (Sambrook, et al., page B-23) containing EtBr. Fragments of gel containing the PCR products were cut out and placed in 2 mL microfuge tubes. The mass of each fragment was determined and 3 µL/mg of Buffer QX1 from QIAquick Gel Extraction Kit (Cat. # 28704 from QIAGEN Inc.) was added. Each tube was then incubated for 10 minutes at 50°C with mixture every two minutes. 1 µL/mg of isopropanol was added to each tube and mixed. The resulting solution was transferred to a QIAquick spin column from the  
15 QIAquick Gel Extraction Kit. These columns were centrifuged for 1 minute at 10 000 g at room temperature. 750 µL of Buffer PE from the QIAquick Gel Extraction Kit were added to each column followed by centrifugation for 1 minute at 10 000 g at room temperature. Each column was centrifuged again for 1 minute at 10 000 g at room temperature to remove any residual buffer. 30 µL of sterile deionized water at 50°C were added to each column and  
20 incubated for 1 minute at room temperature. Each column was then placed in a microfuge tube and centrifuged for 1 minute at 10 000 g.

Next, 6 µL of purified PCR product was transferred to a clean microfuge tube and 1 µL of 10x Ligation Buffer, 2 µL of pCR 2.1 vector and 1uL of T4 DNA Ligase (all from the Original TA Cloning Kit (Cat. # K2000-J10) Invitrogen, Carlsbad, CA) were added to the  
25 microfuge tube and mixed. The reaction was incubated over night at 16°C.

One 50 µL vial of frozen One Shot competent cells (Invitrogen) was thawed on ice for each ligation reaction. 3.5uL of ligation reaction mixture was added to the competent cells

and gently mixed. The cells incubated on ice for 30 minutes, followed by a 30 second “heat shock” at 42°C. After cooling on ice again, 250 µL of SOC medium (Invitrogen) was added to each vial and the vials were put directly into a 37°C water bath for 1 hour. 50 µL and 200 µL aliquots of each transformation reaction were plated on Luria-Bertani Medium (Sambrook et al., page A-1) containing 1% agarose, 1 mg/mL glucose, 50 ng/mL ampicillin and 1.6 ng/mL X-gal. The inoculated plates were then incubated at 37°C overnight. White colored colonies were selected and used to inoculate separate 100 mL cultures of L-B Medium containing 50 ng/mL ampicillin which were incubated at 37°C overnight while being stirred at 100 rpm.

Plasmid DNA was prepared using QIAGEN Midi Plasmid Kit (Cat. # 12143 from QIAGEN Inc.), according to the manufacturer’s instructions, as follows. Each 100 mL culture was aliquoted into two 50 mL conical vials and centrifuged at 3 000 g for 10 minutes at 4°C. The supernatant was poured off and 4 mL of Buffer P1 were used to resuspend the pellet in one of the two vials. This suspension was transferred to the other tube and used to resuspend that pellet. Buffer P2 (4 mL) was added, mixed and the suspension was incubated at room temperature for 5 minutes. Buffer P3 (4 mL) was added, mixed and the suspension was incubated at 4°C for 15 minutes. The samples were centrifuged at 3 000 g for 15 minutes at 4°C. All of the supernatant was transferred to a QIAgen-tip 100 that had been previously treated with 4 mL of Buffer QBT. The supernatant was allowed to drain through the column. Buffer QC (10 mL) was added to the column and allowed to drain. Another 10 mL of Buffer QC was added and allowed to drain. Finally 4 mL of Buffer QF was added to the column and allowed to drain into a clean 15 mL conical vial. 2.8 mL of isopropanol was added and mixed with this solution. This was aliquoted into microfuge tubes, incubated at 4°C for 10 minutes and centrifuged at 10 000 g for 15 minutes at 4°C. The supernatant was removed, then 1 mL of 70% ethanol was added to each microfuge tube. Each tube was vortexed briefly, then centrifuged at 10 000 g for 15 minutes at 4°C. The supernatant was removed and 100 µL of sterile 1x TE buffer (Sambrook et al., page B-20) were added.

Restriction digests were performed on the purified DNA. Enzymes used to cut fragments out were: fragment 1, KpnI (Cat. # R0142S from New England Biolabs, Inc., Beverly, MA) and NsiI (Cat. # R0127S from New England Biolabs, Inc.); fragment 2, NsiI and HindIII (Cat. # R0104S from New England Biolabs, Inc.); fragment 3, HindIII and PstI (Cat. # V0279S from New England Biolabs, Inc.); and fragment 4, PstI and NotI (Cat. #

R0189S from New England Biolabs, Inc.). All restriction digests were preformed at 37°C in digestion buffers recommended by New England Biolabs.

Electrophoresis was performed on all restriction digest products using 10x gel-loading buffer IV (Sambrook et al., page 6.12), on a 1% agarose gel in 0.5x TBE buffer (Sambrook et al., page B-23) containing EtBr. Fragments of gel containing the restriction digest products were cut out and placed in 2 mL microfuge tubes. DNA fragments were extracted from the gel slices using a QIAquick Gel Extraction Kit (Cat. # 28704 from QIAGEN Inc.) according to the manufacturer's instructions. The mass of each fragment was determined and 3  $\mu$ L/mg of Buffer QX1 was added. Each tube was then incubated for 10 minutes at 50°C with mixture every two minutes. One volume of isopropanol was added to each tube and mixed. The resulting solution was transferred to a QIAquick spin column and centrifuged for 1 minute at 10 000 g at room temperature. Buffer PE (750  $\mu$ L) was added to each column followed by centrifugation for 1 minute at 10 000 g at room temperature. Each column was centrifuged again for 1 minute at 10 000 g at room temperature to remove any residual buffer. 30  $\mu$ L of sterile deionized water at 50°C was added to each column and incubated for 1 minute at room temperature. Each column was then placed in a microfuge tube and centrifuged for 1 minute at 10 000 g.

Two microliters of each fragment was added to a microfuge tube as well as 1  $\mu$ L of 10x Ligation Buffer and 1  $\mu$ L of T4 DNA Ligase (Cat. # M0202S from New England Biolabs, Inc.). This reaction was incubated at 16 °C overnight. Restriction enzymes KpnI and NotI (1  $\mu$ L each) were added to the reaction and incubated at 37°C for 1 hour. Electrophoresis was performed using 10x Gel-loading Buffer IV on a 1% agarose gel in 0.5x TBE buffer containing EtBr. The fragment of gel containing the assembled product was cut out and the DNA fragment was extracted using a QIAquick Gel Extraction Kit as described above.

The fragment was ligated into a vector using an Original TA Cloning Kit, as follows: 6  $\mu$ L of purified DNA were transferred to a clean microfuge tube and 1uL of 10x Ligation Buffer, 2  $\mu$ L of pCR 2.1 vector (previously digested with KpnI and NotI), and 1  $\mu$ L of T4 DNA Ligase were added and mixed. The reaction was incubated over night at 16°C. The ligation reaction mixture (3.5  $\mu$ L) was used to transform One Shot competent cells as described above, 50uL and 200uL aliquots of the transformation reaction were plated on Luria-Bertani Medium containing 1% agarose, 1mg/mL Glucose, 50ng/mL Ampicillin and 1.6ng/mL X-gal and grown as described above. White colored colonies were selected and

grown as described above. Plasmid DNA was isolated and purified using a QIAGEN Midi Plasmid Kit as described above. DNA was resuspended in 100  $\mu$ L of sterile 1x TE buffer. This DNA was then sequenced using ABI 100 DNA sequencer (from ABI) by Tufts University Core Facility.

5

*Identification of Four Genotypes of Dog P-glycoprotein*

The dog P-glycoprotein identified in Example 1 is now termed genotype C. Sequencing of the DNA isolated from beagle liver as described in this example permitted the identification of three additional allelic variants of dog P-glycoprotein, termed genotypes A, B and D. the allelic differences in DNA sequence are set forth in Table 1 below.

10

Table 1: Nucleotide and Amino Acid Differences Between Dog P-glycoprotein Genotypes (Allelic Variants)

SEQ ID NO:			Nucleotide position				Amino acid position			
DNA	Protein	Genotype	91	607	1001	3458	25	197	329	1148
22	23	A	T	A	T	A	N	Q	S	M
24	25	B	A	A	T	A	K	Q	S	M
1	2	C	T	C	T	A	N	H	S	M
26	27	D	A	A	A	G	K	Q	T	V

15

Genotype A has a different nucleotide at base number 607 relative to SEQ ID NO:2. This is a base change from C to A in the DNA sequence which causes an amino acid change from Histidine to Glutamine at amino acid 197. Genotype B has different nucleotides at base number 91 and 607 relative to SEQ ID NO:2. The base change at position 91 is from T to A and causes an amino acid change from Asparagine to Lysine at amino acid 25. Base change at position 607 is identical to that in Genotype A. Genotype D has different nucleotides at base number 91, 607, 1001 and 3458 relative to SEQ ID NO:2. Base changes at position 91 and 607 are identical to that in Genotype B. The base change at position 1001 is from T to A and causes an amino acid change from Serine to Threonine at amino acid 329. The base change at position 3458 is from A to G and causes an amino acid change from Methionine to Valine at amino acid 1148.

20

25

A previously identified dog P-glycoprotein (SEQ ID NOs:3 and 4; GenBank Accession number AF045016), has 13 nucleotide differences from the genotype C dog P-glycoprotein nucleotide sequence (SEQ ID NO:1). There is a 3 base deletion at base 89 to

base 91 (relative to SEQ ID NO:1) leaving out AAT which causes a deletion of amino acid 25 Asparagine. There is a base change from A to G at position 590 which causes an amino acid change from Isoleucine to Valine at amino acid 192. There is a base change from C to A at position 607 which causing an amino acid change from Histidine to Glutamine at amino acid 5 197. There is a base change from G to C at position 651 which causes an amino acid change from Arginine to Proline at amino acid 212. There is a base change from G to A at position 878 which causes an amino acid change from Glycine to Arginine at amino acid 288. There is, a base change from T to A at position 1001 which causes an amino acid change from Serine to Threonine at amino acid 329. There is a base change from A to G at position 1012 10 which does not cause an amino acid change. This is a silent point mutation at Glutamine, amino acid 332. There is a base change from A to G at position 1611 which causes an amino acid change from Glutamine to Arginine at amino acid 532. There is a base change from A to T at position 2098 which does not cause an amino acid change. This is a silent point mutation at Valine, amino acid 694. There is a base change from C to T at position 2102 which causes 15 an amino acid change from Proline to Serine at amino acid 696. There is a base change from C to T at position 3808 which does not cause an amino acid change. This is a silent point mutation at Alanine, amino acid 1264. There is a base change from G to A at position 3833 which causes an amino acid change from Valine to Isoleucine at amino acid 1273. There is a base change from C to T at position 4080 which causes an amino acid change from Threonine 20 to Isoleucine at amino acid 1355.

Each of the foregoing patents, patent applications and references is hereby incorporated by reference. While the invention has been described with respect to certain embodiments, it should be appreciated that many modifications and changes may be made by 25 those of ordinary skill in the art without departing from the spirit of the invention. It is intended that such modification, changes and equivalents fall within the scope of the following claims.

What is claimed is: